P-GLYCOPROTEIN CHARACTERISTICS AND INVESTIGATION OF P-GLYCOPROTEIN MEDIATED DRUG-DRUG INTERACTIONS WITH *IN VITRO* METHODS

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Tiivistelmä – Referat – Abstract
P-glycoprotein is an ATP-dependent efflux protein expressed in many tissues which participate in absorption, distribution and elimination of drug molecules. It can mediate clinically significant drug-drug interactions. Characteristics of P-gp have been studied widely and crystal structure of mouse P-gp has been successfully determined. P-gp binds its substrates either directly from cell membrane or from cytosol and it has at least three separate binding sites. P-gp has wide selection of substrates from many therapeutical groups. According to the latest computational models, a typical P-gp substrate can be defined with the help of molecule structural factors rather than physicochemical properties. However function of P-gp is very complex which is why drug-drug interactions should be studied for each drug pair separately. In addition expression of P-gp is regulated by nuclear receptors PXR and CAR thus P-gp induction is separate, which also complicates P-gp mediated interactions.

P-gp substrates celiprolol, talinolol, aliskiren and fexofenadine have *in vivo* interactions with P-gp inhibitors or inducers. The objective the experimental work was to study suitability of two *in vitro* methods, MDCKII-cell permeability assay and MDR1-vesicle transport assay, for predicting *in vivo* effect of drug-drug interaction. ATP-dependent transport of substrates was determined in membrane vesicles extracted from human P-gp expressing Sf9 cells. Cell assay was used to determine efflux ratio (ER) for all the substrates alone and efflux ratio with P-gp inhibitor itraconazole for the substrates which have reported *in vivo* interaction with itraconazole.

All compounds showed ATP dependent transport in MDR1-vesicles and celiprolol, talinolol and fexofenadine showed ER over 1 in MDCKII-MDR1 cells thus according to vesicle assay and ER-value they are P-gp substrates. However ER of talinolol and fexofenadine was not affected by inhibitor itraconazole, thus the drugs did not fulfil the inhibition criteria of FDA for P-gp substrates. The performing of interaction test was possible failed due lack of pre-incubation of the cells with the inhibitor.

Talinolol had the highest ER in thus according to cell experiments talinolol has P-gp dependent transport. Aliskiren ER was not obtained because of the low recovery of the drug but it had clear ATP-dependent transport in the vesicle assay as was expected according to *in vivo* results. According to *in vitro* results and *in vivo* studies celiprolol is a poor P-gp substrate whereas fexofenadine showed P-gp mediated transport both *in vitro* and *in vivo*. The results suggest that significance of drug interaction is difficult to predict with the vesicle and the cell assay but they can be used to recognize P-gp substrates.

Avainsanat – Nyckelord – Keywords
P-glycoprotein/MDRI, transporter, MDCKII-MDR1, membrane vesicles, drug-drug

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Tiivistelmä – Referat – Abstract

P-glykoproteiini on ATP:sta riippuvainen efluksi-kuljetinproteiini joka ilmentyy useissa lääkkeen imeytymiseen, jakautumiseen ja eliminaatioon osallistuvissa kudoksissa ja voi siten aiheuttaa kliinisesti merkittäviä yhteisvaikutuksia. P-gp:n ominaisuuksia on tutkittu paljon ja mm hiiren P-gp:n kiderakenne on selvitetty. P-gp sitoo substraatit joko suoraan solumebraanista tai sytosolista ja sillä on vähintään kolme sitoutumispaikkaa. P-gp:llä on paljon substraatteja monista terapiaryhmistä. Viimeisten tutkimusten mukaan molekyylin rakenne ennustaa paremmin P-gp sitoutumisen kuinfysikokemialliset ominaisuudet. P-gp toimintamekanismi on kuitenkin monimutkainen ja lääke-lääke yhteisvaikutus pitäisi tutkia jokaiselle lääkeparille erikseen. Lisäksi P-gp:n ilmentymistä säätelevät tumareseptrit PXR ja CAR, mikä lisää P-gp:n yhteisvaikutusten monimutkaisuutta.

P-gp substraateille seliprololille, talinololille, aliskireenille ja feksofenadiinille on raportoitu *in vivo* yhteisvaikutuksia P-gp inhibiittoreiden tai indusoijien kanssa. Kokeellisen työn tavoitteena oli tutkia kahden *in vitro* menetelmän, MDCKII-MDR1-solujen ja MDR1 vesikkeleiden soveltuvuutta *in vivo* vaikutuksen ennustamiseen. Substraattien ATP-riippuvaista kuljetusta tutkittiin P-gp:tä ilmentävistä Sf9-soluista valmistetuissa membraani vesikkeleissä. Solukokeissa määritettiin kuljetussuhde yksikerroksisen solukerroksen yli (efflux ratio, ER) jokaiselle substraatille yksin sekä inhibiittorin, itrakonatsolin kanssa niille substraateille, joille on raportoitu *in vivo* yhteisvaikutus itrakonatsolin kanssa.

Kaikkilla yhdisteillä todettiin ATP:sta riippuvaista kuljetusta MDR1-vesikkeleissä ja ER-arvo oli suurempi kuin 1, joten ne ovat P-gp substraatteja vesikkelikokeiden ja ER-arvon mukaan. Kuitenkaan inhibiittorin kanssa tutkituilla lääkeaineille talinololilla ja feksofenadiinilla ER-arvo ei muuttunut inhibiittorin vaikutuksesta, joten ne eivät täytä FDA:n inhibitio-kriteeriä P-gp substraatille. Kokeet mahdollisesti epäonnistuivat, koska soluja ei inkuboitu inhibiittorin kanssa ennen koetta.

Talinololilla oli suurin ER-arvo joten solukokeiden perusteella talinololilla on P-gp riippuvaista kuljetusta. Aliskireenin ER-arvoa ei onnistuttu määrittämään sen alhaisen pitoisuuden vuoksi mutta sillä oli selvästi ATP-riippuvaista kuljetusta vesikkelikokeessa, kuten odotettiin *in vivo* tulosten perusteella. *In vitro* ja *in vivo* tulosten mukaan celiprolol on huono P-gp subtraatti kun taas feksofenadiinilla oli P-gp välitteistä kuljetusta sekä *in vivo* että *in vitro*. Kokeellisen työn tuloksien mukaan lääke-interaktion merkittävyyden ennustaminen on vaikeaa käytetyillä tutkimusmeneltemillä, mutta niitä voidaan käyttää P-gp subtraattien tunnistamiseen.

Avainsanat – Nyckelord – Keywords

P-glykoproteiini, MDRI, kuljetusproteiini, MDCKII-MDR1, membraani vesikkelit, lääke-lääke yhteisvaikutus

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I LITERATURE REVIEW

1 INTRODUCTION

Human multidrug resistance protein 1 (MDR1), commonly known as P-glycoprotein (P-gp) is an efflux transporter. It was first discovered in 1976 from colchicine resistant Chinese hamster ovary cells (Litman et al. 2001). P-gp is coded by gene *ABCB1* and belongs to ATP-binding cassette (ABC) transporters superfamily which, according to their name, functions by utilizing energy from ATP. P-gp is found in epithelial cells of tissues such as intestine, liver, kidneys, brain, testis, placenta and lung and it is expressed in many cancer cells (Litman et al. 2001, Leslie et al. 2005). Recently it was discovered also in ocular tissue (Senthilkumari et al. 2008). P-gp is located in cell membrane where it transports its substrates away from the cell interior. Its physiological role is to protect vital tissues such as brain and testis by preventing the entry of harmful compounds (Sharom et al. 2005). Another role is to promote removal of compounds from the body by transporting them to bile, urine and intestine (Figure 1). P-gp can affect drug absorption, distribution and elimination and mediate drug-drug interactions and therefore greatly affect the safety and efficacy of P-gp substrates (Giacomini et al. 2010).

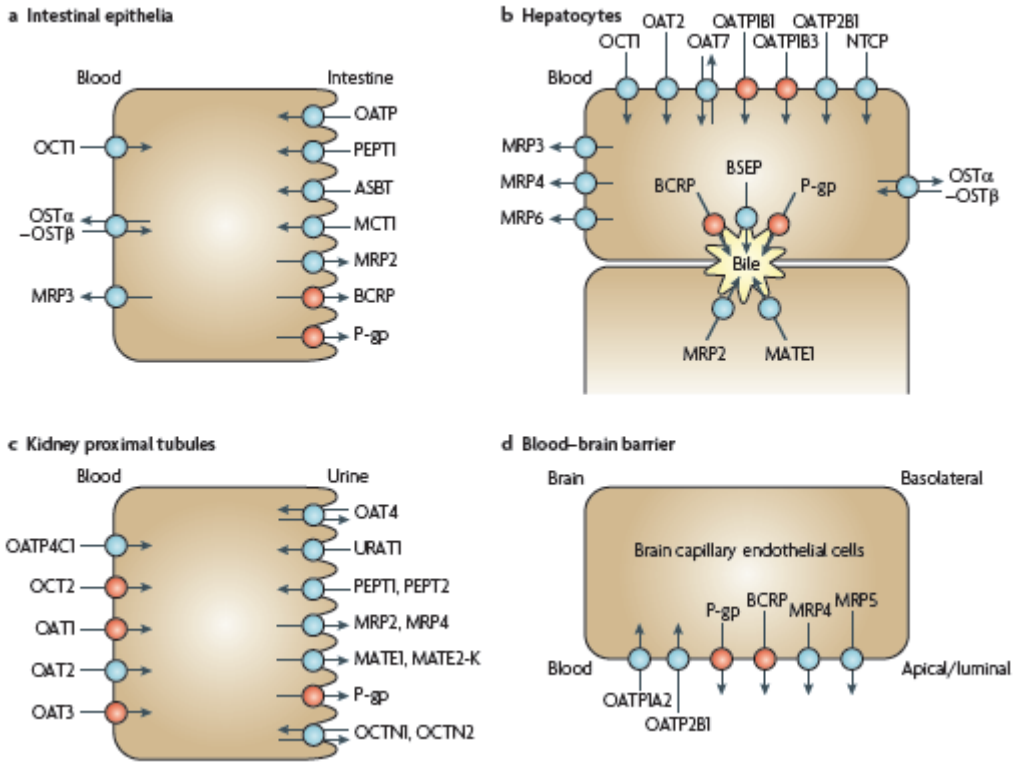


Figure 1. Location of transporter proteins and direction of transport in intestinal epithelia, hepatocytes, kidney proximal tubules and blood-brain barrier. P-glycoprotein transports substrates out from the cell in the apical membranes of intestine and blood-brain barrier, and to the urine and bile in the kidney proximal tubule and hepatocytes, respectively. (Giacomini et al. 2010).

P-gp is known to transport a wide selection of drugs and other compounds. During drug development it is vital to avoid compounds which may behave unexpectedly in the body and cause safety problems (EMA 2012). However, often a new drug molecule is found to be a transporter substrate after releasing to market. This problem could be solved if the structure and function of the protein is understood. If characteristics of substrate and its binding place are known, structures which are recognized by the protein could be avoided in new drug molecules. Also resistance of P-gp substrates could be overcome in theory by developing compounds which inhibit the function of protein (Stouch and Gudmundsson 2002). Understanding the mechanism of function and regulation of P-gp also makes it possible to control these processes (Bartolini et al. 2006).

In this literature survey the structure of P-gp, mechanism of function, and regulation are reviewed. This is done to understand backgrounds of P-glycoprotein mediated drug-

drug interactions. Also substrate selection and structure of P-gp substrates is viewed to find out if it is possible to characterize typical P-gp interacting molecule.

2 STRUCTURAL CHARACTERISTICS OF P-GLYCOPROTEIN

2.1 Structure of P-glycoprotein

Human P-gp is a rather large protein composed of 1280 amino acids and weighting 170 kDa (Hennessy and Spears, 2007). A 150 kDa P-gp intermediate is formed in endoplasmic reticulum and further glycosylated prior exporting to the cell surface. The protein seems to be formed by duplication of genes and it has two halves which both have 6 α-helices comprising trans-membrane domain (TMD) and nucleotide-binding (ATP-binding) domain (NBD) (Hennessy and Spears, 2007, Jin et al. 2012). P-gp is located in cell membrane with most of the molecule inside the membrane as shown in Figure 2. The NBD is facing interior of the cell.

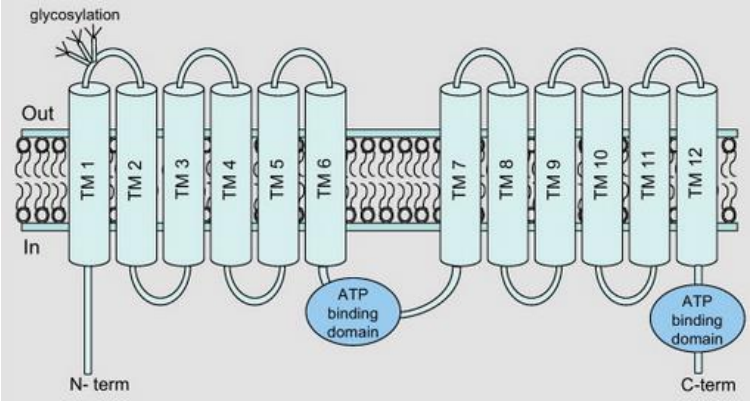


Figure 2. P-glycoprotein is located in the cell membrane with ATP (adenosine triphosphate) binding domains facing the interior of the cell. It comprises two halves which both have six trans-membrane (TM) α-helices (Fu and Arias 2012)

So far the crystal structure of human P-gp has not been obtained but X-ray crystal structure of mouse P-gp (Figure 3) and *Caenorhabditis elegans* (*C.elegans*) PGP-1 have been determined (Aller et al. 2009, Jin et al. 2012). With the help of crystal structure the

dimensions of the protein and position of TM helices can be clarified. P-gp crystal structure confirms commonly accepted idea that the two halves of the protein are formed from six trans-membrane helices (Aller et al. 2009, Jin et al. 2012). With the mouse P-gp the one half is formed from TM1, 2, 3, 6, 10 and 11 and the other from TM4, 5, 7, 8, 9 and 12 leaving roughly 6000 $Å^2$ a cavity between the two halves of protein (Aller et al. 2009). The height of total protein is about 70 Å, length 136 Å and distance between NB-domains is about 30 Å.

The protein is "open" to the inside of the cell membrane leaflet and also to the cytosol (Rosenberg et al. 2005, Aller et al. 2009, Jin et al. 2012). The binding of P-gp substrates is assumed to happen in the "binding cavity" left between two halves of protein (Aller et al. 2009, Jin et al. 2012). Aller et al. (2009) suggested that the crystallized form of mouse P-gp is in the substrate binding state and can open even wider than illustrated in the crystallized form. Large size of the protein and shape of the "binding cavity" enables binding of large molecules or more than one molecule at the same time which could partly explain why P-gp has a wide selection of substrates and ability to bind several molecules at the same time.

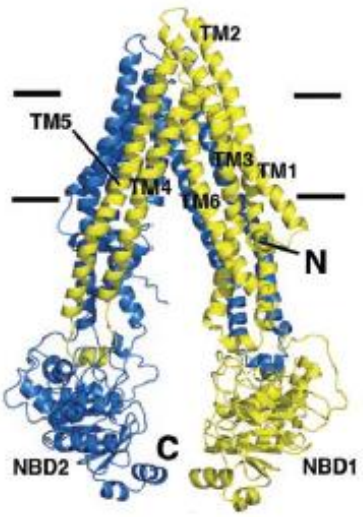


Figure 3. X-ray crystal structure of mouse P-gp-protein. A binding cavity is formed between the two halves (presented with blue and yellow) of the protein. NBD is nucleotide binding domain and TM is transmembrane domain (modified from Aller et al. 2009).

The nature of amino acid residues predicts the ability of molecules with variable hydrophobicity to interact with the protein. Most of the amino-acids facing inside in the upper part of the cavity are hydrophobic or aromatic which makes the binding cavity hydrophobic and easier for lipophilic molecules to get access to (Aller et al. 2009). However in the lower part of the cavity there are also hydrophilic residues and therefore also polar compounds can get inside the cavity. Loo et al. (2004) further suggested that there is an aqueous environment in the binding cavity. The group demonstrated that cysteines in TM5 can interact with charged compounds (thiol-reactive compounds MTSES and MTSET). They proposed that hydrophobic substrates could be hydrolyzed prior entering the cavity and hence stabilized to stay in the cavity.

The amino acid structure of mouse P-gp is 87 % and *C. elegans* PGP-1 46 % similar to human P-gp thus the structure of human P-gp is probably different from the structures of the two species. At least two trans-membrane helices of mouse P-gp are different compared to human P-gp and ATPase activity of *C. elegans* PGP-1 was stimulated by only part of the human P-gp substrates (Jin et al. 2012, Li et al. 2013). On the other hand according to Li et al. (2013) the sequence identity of amino acids in drug pathway is rather high between mouse and human P-gp (96 %) while *C.elegans* sequence identity of PGP-1 and human P-gp is much lower (13 %). Thus the crystal structure of mouse P-gp serves as a good model for the P-gp fine structure until the crystal structure of human P-gp is determined.

2.2 Ligand binding sites

Relevant areas for drug binding have been studied by inducing mutations in certain amino acid residues. Ambudcar et al. (1999) reviewed extensively the effect of mutation in different trans-membrane domains (TM), extracellular loops (EC), intracellular loops (IC), and nucleotide binding domains (NBD) on substrate activity. Mutation in several human P-gp TM-helices (Table 1.) as well as in mouse TM11 had an effect on substrate binding. Also mutation in EC1, IC1, and 4, and N-NBD had an effect on P-gp function. However it must be noted that mutation can affect the protein function also by changing the shape of the protein. According to more specific method based on direct binding of

thiol-analogues with TM cysteine residues the binding pocket of human P-gp is at least partly formed from TMs 1, 6, 7, and 12 (Table 1) (and Clarkeal. 2006a, Loo et al. 2006b, Loo and Clarke 1997).

Table 1. Essential transmembrane domains (TMs) for substrate binding of human P-glycoprotein determined with mutation analysis and thiol-analogue binding methods.

Analysis method	TMs (human P-gp)
Mutation analysis[1]	1, 4, 5, 6, 10, 12
Thiol-analogues[2]	1, 6, 7, 12

[1] Ambudcar et al. 1999
[2] Loo et al. 2006a, Loo et al. 2006b, Loo and Clarke 1997

A protein is a dynamic complex which is why it is difficult to separate the most important TM-helices. However TMs 6 and 12 are the most frequently considered as essential for substrate-protein interaction. Mutation in TM6 or nearby TM6 of humans P-gp can alter the effect of P-gp on its substrates (vinblastine, colchicine, doxorubicin and actinomycin) and change the activity of a P-gp inhibiting monoclonal antibody UIC2 (Loo and Clarke 1994a, Zhou et al. 1999). Also in crystal structure complex of mouse P-gp and a P-gp inhibitor QZ59 (Figure 4.) the compound is located in the intracellular side of the protein, close to the TM6 and TM12 (Aller et al 2009). Two tested isomers (QZ59-SSS and QZ59-RRR) had some differences in orientation and location of the compounds in the cavity but for example the isopropyl groups of the both molecules were close to TM 12 (and 9).

The crystal structures of both mouse and *C.elegans* protein indicate that protein α-helices are at the same angle with each other except for one pair, TMs 6 and 12 and TMs 10 and 12, respectively (Aller et al 2009, Jin et al. 2012). Also Rosenberg et al. (2005) found that in 3D-structure (obtained from 2D crystals) of Chinese hamster P-gp there are non-organized helices TM 6 and TM 12. More open helixes have readily interactions with possible ligands and other surrounding molecules thus they probably participate in binding of ligands and directing the substrates to the trans-membrane cavity (Rosenberg et al. 2005, Jin et al. 2012). This also confirms importance of TMs 6 and 12.

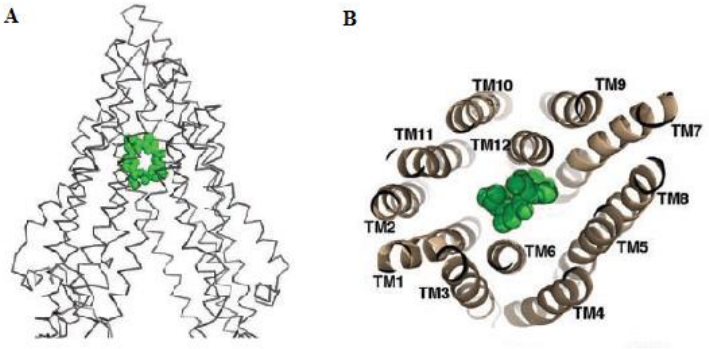


Figure 4. Crystal structure of mouse P-glycoprotein binding cavity hosting a P-gp inhibitor QZ59-RRR A) from plane and B) from above. The compound is located in the intracellular side of the protein, close to the transmembrane domains 6 and 12 (modified from Aller et al 2009).

Having many of the TM helices interacting with substrates indicates a high binding potential. At least two separate binding sites for P-gp substrates have been proposed by several research groups (Dey et al. 1997, Shapiro and Ling 1997a, Shapiro and Ling 1997b, Shapiro and Ling 1998, Loo et al. 2003c, Wang et al. 2003). Shapiro and Ling (1997b) demonstrated that fluorescent agents Hoechst 3342 and rhodamine 123 stimulated each other's P-gp mediated transport and were transported at the same time in Chinese hamster ovary cell vesicles which indicates that molecules bind to different binding places. The two binding sites were named R- (rhodamine 123) and H- (Hoechst 33342) site according their substrates. It was also demonstrated that mutation in one TM-helix (TM5, which is close to binding site of verapamil) decreased ATPase activity stimulated by verapamil and vinblastine but there was no effect on activation by rhodamine B and colchicine indicating that the latter molecules bind to different site than verapamil and vinblastine (Loo et al. 2004). Recently the successful co-crystallization of mouse P-gp with two substrate (QZ59-SSS**)** molecules further demonstrated that P-gp can in fact occupy at least two molecules at the same time (Figure 5) (Aller et al. 2009).

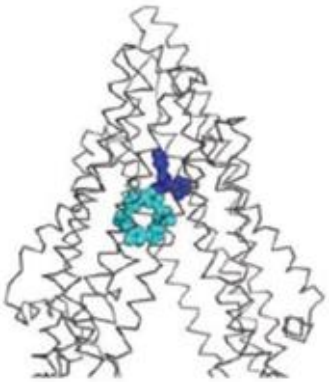


Figure 5. Mouse P-gp can host two molecules of a P-glycoprotein substrate QZ59-SSS at the same time in the internal cavity of the protein (modified from Aller et al 2009).

With the help of specific R- and H-site substrates it was demonstrated that the two binding sites have different substrate selectivity. Transport rate of Hoechst 33342 and Rhodamine 123 was affected in different manner by P-gp substrates (Shapiro and Ling 1997b). The same molecules that increased the transport rate of rhodamine 123 in concentration dependent manner in P-gp-expressing vesicles decreased the transport rate of Hoechst 33342. Thus it seems that one part of the molecules bound to the R-site and other part bound to the H-site. Another option is that there are even more than two binding sites (Shapiro et al. 1999, Martin et al. 2000). Shapiro and coworkers demonstrated that the transport rate of Hoechst 33342 was stimulated additively in plasma membrane vesicles if it was administered with both Rhodamine 123 and prazosin or progesterone. On the other hand simultaneous administration of prazosin and Hoechst 33342 did not stimulate transport rate of rhodamine 123, but seemed to interfere the additive effect of Hoechst 33342. The group suggested that prazosin and progesterone bind to third, allosteric binding site which has different kind of effect on H- and R-site. The function of P-glycoprotein binding sites based on specific H- and R-sites is concluded in Figure 6. Martin et al. (2000) further suggested that P-gp could have even four different binding sites for ligands, of which one acts solely as a regulatory site whereas the H-site has both regulatory and transport function.

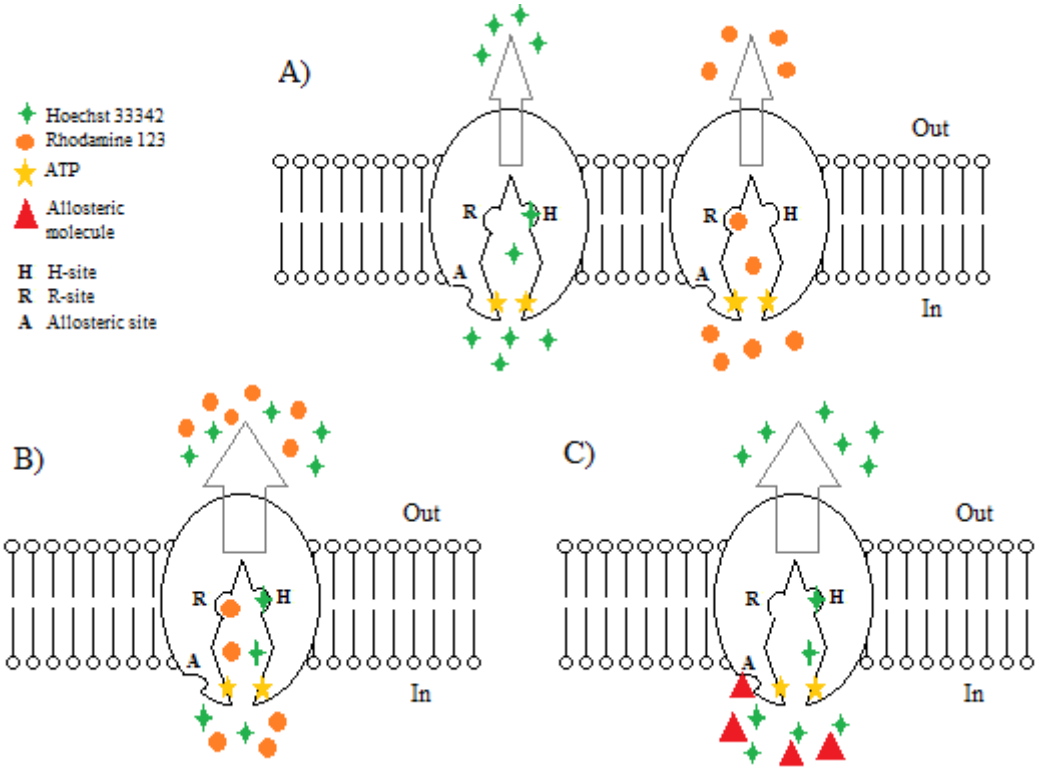


Figure 6. P-glycoprotein has two binding sites (H- and R-site) which can function simultaneously and additively and an allosteric binding site A) Hoechst 33342 binds to H-site and Rhodamine 123 to the R-site to be transported. B) When both molecules are present they increase each other's transport rate C) Binding to allosteric site increases transport of Hoechst 33342. Arrow indicates transport rate.

3 FUNCTION OF P-GLYCOPROTEIN

3.1 Binding of ATP

It is clear that ATP hydrolysis is required for transporting P-gp substrates (Al-Shawi and Senior 1993, Kimura et al. 2004). As mentioned before P-gp has two nucleotide binding sites which are hydrophilic and located in cytosol (Rosenberg et al. 2005). Mutation in NB-domain of at least N-terminal affects the function of the protein (Hoof et al. 1994). The NB-domains have also several motifs, (such as Walker A, Walker B, LSGGQ/ABC signature motif) which participate in ATP hydrolysis and interact with ATP (Walker et al. 1982, Hennessy and Spears 2007). Both NBs have been

demonstrated to be active yet dependent on each other (Urbatsch et al. 1995). In early studies it was found that N-terminal NB site hydrolyzed ATP without interaction with the C-terminal (Shimabuku et al. 1992). Later studies however implicate that both ATP molecules have role in the catalytic cascade which causes transport of substrate (Dey et al. 1997, Sauna et al. 2007, Wang et al. 2003).

P-gp has been proposed to act like a two-cylinder engine whose two ATP binding sites hydrolyses ATP molecules and two drug binding sites transports substrates by turns (van Veen et al. 2000). Several studies indicate that there are some conformational changes in P-gp structure after ATP is bound. According to theory of catalytic cycle of ATP hydrolysis, nucleotide binding domains are dimerized by the effect of ATP (Sauna et al. 2007) (Figure 7.). While ATP catalyzes dimerization, ADP causes dissociation of dimer. ATP binds to both nucleotide binding sites but is hydrolyzed at different time at the two binding sites. This cycle causes conformational change of the P-gp molecule and may alter the affinity of the substrates to P-gp.

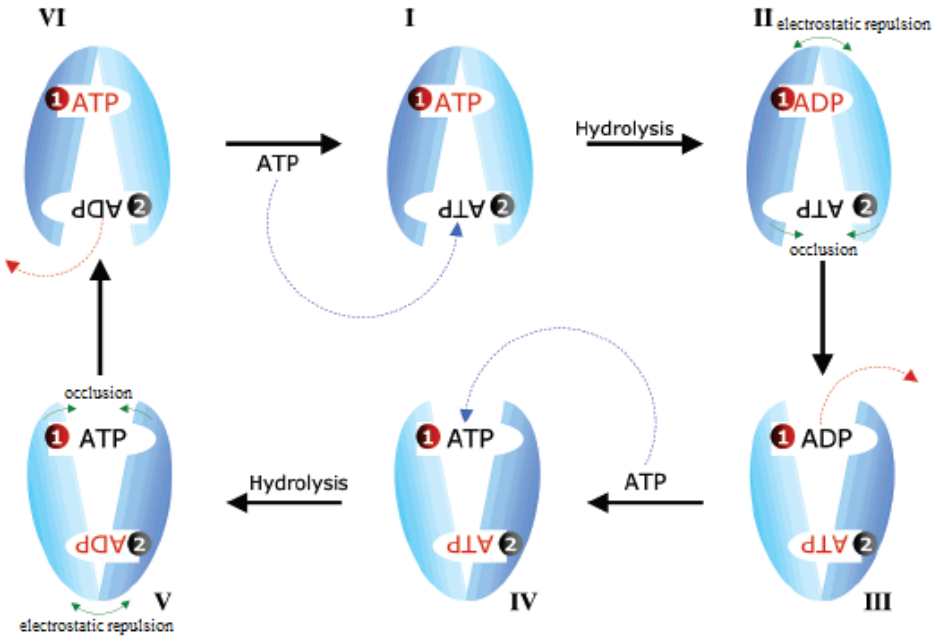


Figure 7. Two ATP molecules catalyze dimerization of P-glycoprotein by turns and causes conformational change in P-glycoprotein structure. One of two ATP molecules is occluded to ATP site (step I) and hydrolyzed to ADP (step II) which causes electrostatic repulsion and simultaneous release of ADP and occlusion of ATP to ATP site 2 (step

III). The cycle (steps I-III) is repeated in APT site 2 (steps IV-VI) (modified from Sauna et al. 2007)

It has been proposed that substrate affinity to two binding sites can alter from low to high affinity state (Dey et al. 1997, Wang et al. 2003). Dey et al. (1997) proposed that the two binding sites of P-gp are "ON" (C-terminal) and "OFF" (N-terminal) site. They suggest that substrates bind more favorable to the "ON" site and moving from the "ON"-site to the "OFF"-site demands the energy from ATP hydrolysis and leads to transport of the substrates. The catalytic cycle of P-gp is explained more closely in Figure 8. Replacement of Pi in Pg-MgADP complex with vanadate (vi) was able to stabilize the complex and prevent substrates from binding to P-gp implicating that both ATP molecules are needed for the transport cycle. This finding and theory of Sauna et al. (2007) confirms that both ATP molecules are needed for the transport cycle.

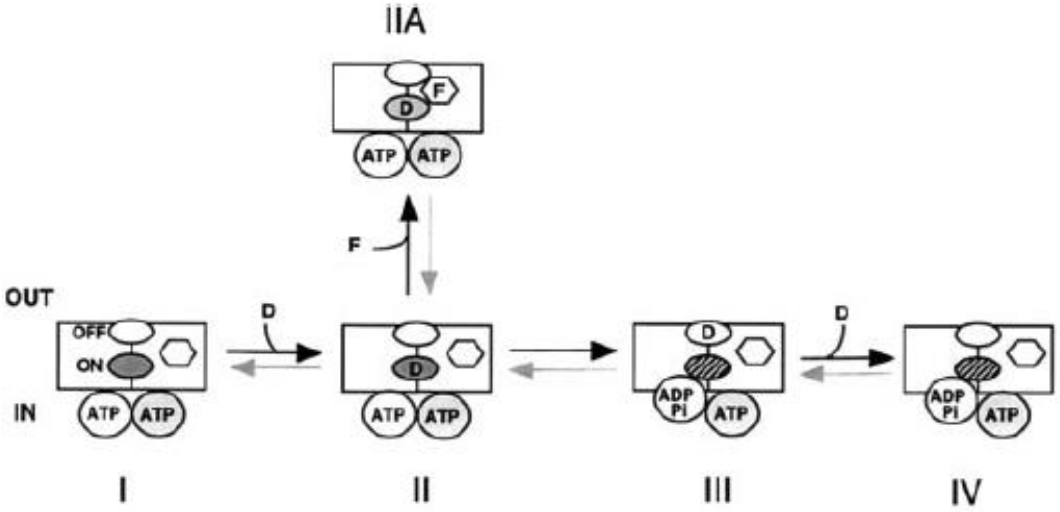


Figure 8. In the catalytic cycle of P-glycoprotein binding sites alter from high to low affinity in different phases of ATP hydrolysis. Squares represent TM (transmembrane) domains, circles ATP-binding sites and ovals substrate interacting sites. Step I: no drug is bound. Step II: drug can bind to the "ON"-site without demanding energy from ATP. Step III: affinity of substrate to the "ON"-site is decreased in consequence of conformational change catalyzed by ATP-hydrolysis and a drug moves to the "OFF"-site. Step IV: drug is released from the "OFF"-site to the exterior of the cell (Adapted from Dey et al. 1997).

3.2 Transport mechanism

The mechanism by which P-gp recognizes and transports its substrates has puzzled researchers and there are at least three different theories for the mechanism of function (Ambukar et al. 1999, Kimura et al. 2004, Sharom et al. 2005). P-gp has been proposed to recognize the substrates directly from membrane because of the lipophilic nature of the substrates (Shapiro and Ling, 1997a). Both H- and R-site substrates have been demonstrated to be transported directly from the membrane cytoplasmic leaflet to the extracellular medium (Shapiro and Ling, 1997a, Shapiro and Ling 1998). Lipophilic drugs can easily dissolve in membranes and may occur with higher concentrations in membranes than in aqueous cytoplasm or extracellular material which also supports the theory that substrates are transported directly from the membrane.

The first theory was that P-gp acts as a "hydrophobic vacuum-cleaner" which transports the substrates immediately when they enter the membrane (Raviv et al. 1990). According to this theory transport is more dependent on the lipophilic nature of substrate than selective binding of substrate. As mentioned before the common opinion is that P-gp substrates are bound by TM-domains (Ambudcar et al. 1999, Shapiro et al. 2005). Substrates could get inside P-gp and "vacuum-cleaned" from the "gates" which are left between the two halves of the protein in the membrane (Figure 9). The gateway for substrates have been suggested to form from TMs 2, 11, 5, and 8 in human P-gp (Loo and Clarke 2005) and TMs 4, 6, 10 and 12 in mouse P-gp (Aller et al. 2009).

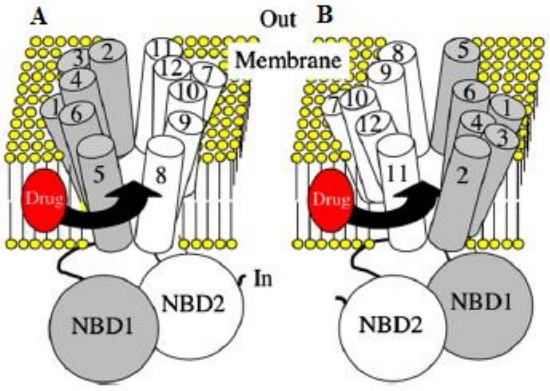


Figure 9. TMs 5 and 8 (A) and TMs 2 and 11(B) form gateway for drug enter to the binding cavity of human P-glycoprotein (Modified from Loo and Clarke 2005)

Efficient transport and hydrophilic nature of substrates have been explained also with another theory. P-gp has been proposed to act as a “flippase” and transport its substrates from the inner leaflet of cell membrane to the outer leaflet of the cell membrane in the same manner its “flips” short chain phospholipids across the lipid bilayer (Higgins and Gottesman 1992, Romsicki and Sharom 2001). It has been proven that P-gp can act as a flippase and translocate hydrophobic fluorescent labeled glycosphingolipids (GSL), sphingomyelin (SM) and cholesterol in the lipid bilayer from one side to another (Garriques et al. 2002, Eckford and Sharom 2005). Like transport of ligands, flipping of phospholipids required presence of ATP and is inhibited by the effect of vanadate which traps P-gp into a transition stage (Romsicki and Sharom 2001, Eckford and Sharom 2005). With the help of FRETT (fluorescence resonance energy transfer), the position of P-gp substrate LDS-751 in the membrane has been demonstrated to be in the cytoplasmic leaflet of the membrane (close to NBs) from where the flipping happens (Lugo and Sharom 2005). In addition P-gp substrates and modulators, such as cyclosporine, verapamil, vinblastine, and PSC-833, inhibited flipping of phospholipids thus flippase function and ligand binding seems to occur at the same site and may be competitive (Romsicki and Sharom 2001). Johnstone and coworkers (2000) proposed that flipping is a fast process whereas the release of substrate from the inner leaflet is slower which explains the high transport rate of P-gp.

Substrate binding (along with binding of ATP) to the protein is the first stage of the transport mechanism but another question is what happens after that. Several theories suggest that the substrate is transported in consequence of conformational change of the protein (Loo and Clarke 2002, Loo et al. 2003a, Aller et al. 2009). This can be concluded also from ATP binding theories presented above which suggest that shape of the protein changes as a consequence of ATP binding and hydrolysis. An “induced fit” mechanism was introduced which proposes that binding of substrate changes the conformation of P-gp which causes the drug transport out of the cell (Loo and Clarke 2002, Loo et al. 2003a).

Already in 1992 Bruggeman and coworkers suggested that two halves approach each other in order to bind a substrate. Aller and coworkers (2009) suggested that P-gp is

open to cytosol in substrate binding state (Figure 10A). After ATP is bound to the NBDs the conformation of protein is changed to the form which is open to the extracellular space (Figure 10B). Conformation change could be result of different cross-linking between TM-domains (Loo et al. 2003a). Cross-linking between TM6 and TM 11 or TM 12 was induced by the effect of P-gp substrates. It was proposed that the conformational change could create a funnel for substrates to move to the other side. The effect of the substrates varied in creating slightly differing cross-links which could be result from binding to different sites. The conformational change of the protein could also create new binding sites for substrates which could explain the great variety of ligands.

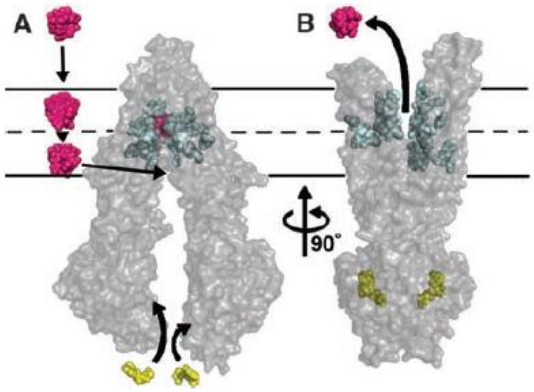


Figure 10. P-glycoprotein is open to cytosol in substrate binding state and after binding of ATP changes the conformation to form which is open to the extracellular space (adapted from Aller et al. 2009)

Different P-gp substrate transport mechanisms are demonstrated in Figure 11. Theory of hydrophobic vacuum cleaner and induced fit are in the same line with each other. The substrate is taken inside the cell by passive absorption (or active transport) and from cytosol to the binding site of P-gp. Alternatively P-gp interacts with the substrate already in lipid bilayer and substrate binds to the binding site. The substrate binding changes the protein conformation which leads to the substrate transport outside the cell. However it is unclear if a flippase mechanism is entirely separate mechanism or does it also require conformational change.

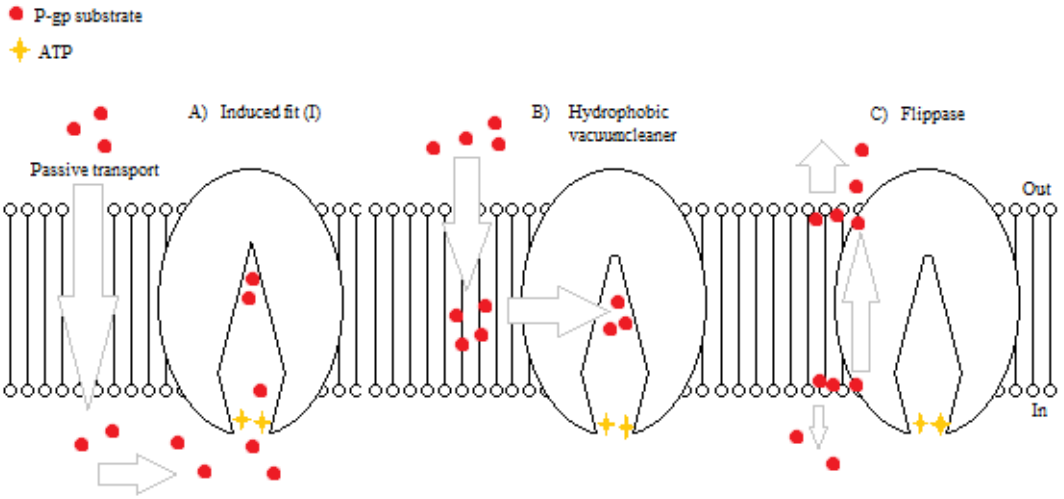


Figure 11. Transport mechanisms of P-glycoprotein (P-gp) substrates A) Induced fit. Substrate binds to the protein and is releases to the extracellular space after conformational change of P-gp B) Hydrophobic vacuum cleaner. Substrate is absorbed to the cell membrane and immediately taken in by P-gp and transported (in the consequence of induced fit) C) Substrate is flipped from the inner membrane leaflet to the outer leaflet by P-gp

3.3 Mechanism of inhibition

Understanding the mechanism of inhibition is of great interest because of the possibility of finding chemo-sensitizers which could improve the efficacy of cytotoxic agents (Ambudcar et al. 1999, Hennessy and Spears 2007). Binding of several substrates on P-gp have been demonstrated to be co-operative, competitive or non-competitive (Acharya et al. 2006, Garrigos et al. 1997, Litman et al. 1997, Shapiro and Ling 1997a; Shapiro and Ling 1997b; Shapiro and Ling 1998). Competitive inhibitors can inhibit the interaction of another ligand with protein by binding to the same binding site or alternatively to the distinct allosteric binding site (Garrigos et al. 1997). Non-competitive inhibitors bind to distinct sites which do not communicate with each other and therefore synergetic effect is possible. For instance vinblastine and cyclosporine inhibited ATPase activity of verapamil or progesterone in competitive manner in P-gp expressing cells whereas verapamil and progesterone had co-operative effect on ATPase activity (Garrigos et al. 1997, Litman et al. 1997). Having several binding sites means that substrates and inhibitors can bind to different sites which explains why different

drugs affect in different manner to P-gp substrate transport. In addition the inhibition of substrate transport could happen by preventing the substrate-binding place interaction but also by interfering the binding or reaction of ATP (Ambudcar et al. 1999).

The complexity of inhibition mechanisms makes it difficult to predict the significance of drug-drug interactions. When the efflux of P-gp substrates and inhibitors was studied in MDCKII-MDR1 cells, P-gp was demonstrated to transport both inhibitor and substrate simultaneously (Acharya et al. 2006). The presence of inhibitor or another substrate did not affect the efflux of substrate at low concentration which can be result from binding to separate places. On the other hand, when the authors fitted rate constants to a model expecting that substrates and inhibitors compete for one binding site, substrates fitted well with high inhibitor/substrate concentrations. The authors proposed that both substrates and inhibitors can bind to the same binding place because the competitive binding occurs when concentration of either rises. According to these studies it seems that there are no specific inhibiting binding sites but inhibition is rather dependent on the structure of certain molecules at the same site. This means that every drug pair should be studied to find out if inhibition occurs.

4 REGULATION OF P-GLYCOPROTEIN

4.1 Factors effecting P-glycoprotein expression

Not only inhibition but also induction of P-gp can cause drug-drug interactions which is why it is important to understand the mechanism which leads to increased protein levels. The regulation of P-gp expression is very complex and can be affected by both environmental stimulus and transcriptional factors (Hennessy and Spears 2007). Especially mechanisms by which chemotherapeutic agents stimulate the expression of P-gp have been of great interest. The phase of the cell proliferation may have some influence on P-gp expression and multidrug resistance (Wartenberg et al. 2002). The group of Wartenberg (2002) studied if the inhibition of the cell cycle and cell cycle

inhibitors affect P-gp expression and multi drug resistance. They demonstrated that the amount of P-gp was increased when largest number of cells was in the resting phase.

It is not clear whether P-gp induction is mediated in receptor level or in gene level (Hennessy and Spears 2007). There is also evidence that some chemotherapeutic drugs can cause epigenetic changes in P-gp coding (MDR1) gene promoter and therefore alter the phenotype of P-gp (Baker et al. 2005). This means that drug resistance is not caused only by increasing transcription of gene but also in the way transcription is regulated. Epigenetic changes could also explain the inter-individual differences in P-gp mediated efflux and gene polymorphism (Lown et al 1997).

4.2 Nuclear receptors

Nuclear receptors pregnane X (PXR) (also known as SXR, NR1I2) and constitutive active receptor (CAR) (NR1I3) have been connected to expression of several metabolizing enzymes (CYP) and transporters (such as P-gp, Mpr2, BCRP) (Dussault et al. 2001, Luo et al. 2002, Zhang et al. 2007, Johnson et al. 2008, Wang et al 2010). Being an orphan receptor, PXR forms a heterodimer with Retinoid X receptor (RXR) and RXR ligands (retinoids) can activate PXR (human or rabbit)-RXR heterodimer thus also involvement of RXR in regulation of P-gp expression is likely (Jones et al. 2000, Sulová et al. 2008). The activation mechanism of nuclear receptors is presented in Figure 12. When a ligand is not present, receptor is in multi-protein complex in cytoplasm (Kliewer et al. 1998, Timsit and Negishi 2007, Slosky et al. 2013). When a ligand appears the receptor is released from the complex and transports into the nucleus. In the nucleus it forms heterodimer with RXR and induces gene transcription.

In consequence of receptor activation by P-gp inducer the expression of mRNA of P-gp and therefore expression of P-gp increases (Lowes et al. 2010). The increased P-gp expression increases the efflux of P-gp substrate and therefore smaller amount of drug can get to the binding place. Some drugs such as paclitaxel can act as their own efflux inducers and therefore prevent their own therapeutic effect (Synold et al. 2001).

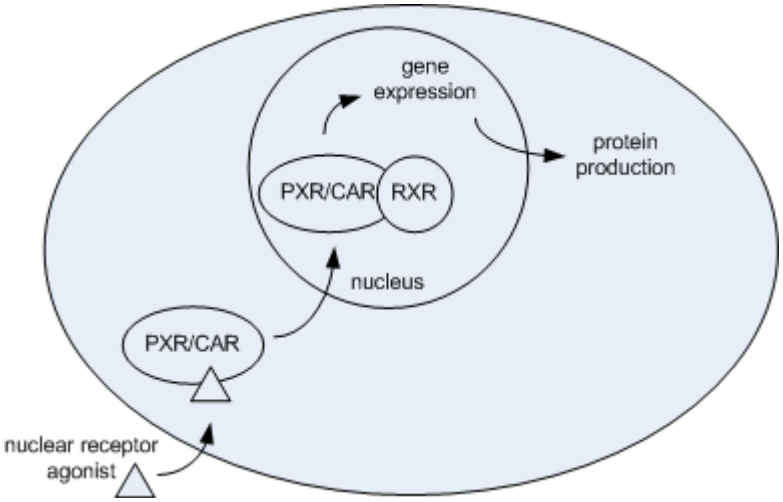


Figure 12. Activation mechanism of nuclear receptors. Pregnane X receptor (PXR) or constitutive active receptor (CAR) receptors bind their substrates in the cytosol, translocate to the cell nucleus and form a heterodimer with RXR. The dimer causes induction of gene expression and protein production.

PXR and CAR receptor expression in the body is somewhat the same as P-gp expression which also implicates that nuclear receptors are involved in P-gp regulation. mRNA of PXR was expressed in high levels in the liver and intestine and with lower levels in the kidney, stomach, ovary, and uterus of an adult mouse (Kliewer et al. 1998, Masuyama et al. 2001). In human tissue mRNA of PXR –receptor was expressed at high levels in the liver, small intestine, and colon and lower levels in other tissues (Nishimura et al. 2004). CAR-receptor levels were the highest in the liver and the kidney. PXR is also expressed in tumor cells where P-gp expression is typically high (Synold et al. 2001).

4.3 Pregnane X receptor

According to the present knowledge nuclear receptor pregnane X (PXR) is an important link in P-gp regulation because known P-gp inducers such as rifampicin, vinblastine and carbamazepine can activate the PXR receptor (Geick et al. 2001, Wentworth et al. 2000, Synold et al. 2001, Luo et al. 2002, Sulová et al. 2008). The mechanism of receptor involvement in P-gp regulation has been studied with P-gp expressing cancer cell lines such as LS180 and LS174T (both from human colon carcinoma) and L1210/VCR

(mouse leukemia) and with different PXR transfected cell lines (such as CV-1) (Geick et al. 2001, Sulová et al. 2008, Harmsen et al. 2012). Multidrug resistance by P-gp overexpression can be induced in L1210 cells with doxorubicin and vincristine and overruled with P-gp-inhibitors (Boháčová et al. 2006). Recently it was suggested that that quickly developing drug resistance is mediated by PXR-receptors because tyrosine kinase inhibitors (TKIs) induced P-gp in LS180 cells (Harmsen et al. 2012). Just in last year it was shown that PXR is involved in regulating P-gp expression also in brain-blood barrier (Chan et al. 2013). When studied with brain microdialysis scientists showed that PXR ligand and P-gp inducer dexamethasone decreased the concentration of P-gp substrate quinidine in brains and up-regulated P-gp expression.

Studying the effect of St. John's wort and its ingredients to expression of PXR proved that P-gp inductive effect is caused by direct interaction of inducer and receptor (Moore et al. 2000, Wentworth et al. 2000). According to present knowledge St. John's wort has an inductive effect on intestinal P-gp along with CYP3A4 (Schwarz et al. 2007). Moore et al. (2000) demonstrated that hyperforin, one of the active ingredients of St. John's wort activated PXR-receptor in CV-1(human hepatocyte). When studied with competition binding it was demonstrated that hyperforin competed with radioactive PXR ligand (SR12813) which proves that hyperforin activates PXR by direct binding to the PXR. Also Wentworth et al. (2000) demonstrated 5-fold difference in activity of PXR with and without St. John's Wort in JEG-3 cells and that in fact, the effect was mediated by binding to the ligand binding domain (LBD) of PXR.

4.4 Constitutive active receptor

Another nuclear receptor, constitutive active receptor (CAR) has been suggested to mediate P-gp induction (Burk et al. 2005, Johnson et al. 2008, Wang et al 2010, Chan et al. 2011, Slosky et al. 2013). Burk et al. (2005) demonstrated that CAR was able to bind to the same gene motif (DR4(I)) of P-gp than PXR. Also when studied in human brain microvessel endothelial cell culture (hCMEC/D3) the expression of both P-gp and P-gp mRNA increased after hCAR and hPXR activation (Chan et al. 2011).

P-gp expression has been connected to the activation of CAR receptor in BBB with rodents (Wang et al 2010, Slosky et al. 2013). The expression of P-gp can be stimulated with CAR ligands (TCPOBOP, phenobarbital) and expression of P-gp mRNA correlates with CAR activation (Wang et al. 2010). Slosky et al. (2013) demonstrated *in vivo* with rats that paracetamol mediated P-gp expression increase in BBB was inhibited by CAR inhibitors (OA and Act D which inhibit translocation of CAR to the nucleus and transcriptional effect, respectively). CAR expression in the nuclear compared to cytosol was measured to demonstrate CAR activation.

It seems evident that P-gp gene regulation is mediated by nuclear receptors. However, the mechanism which leads to gene transcription of certain protein is unclear. As stated before PXR and CAR mediate up-regulation of several transporters and enzymes. Studies demonstrate that enzymes and transporters, such as CYP3A4 and P-gp can have overlapping substrates and interplay (Luo et al. 2002). This is probably at least partly caused by a common regulation system. However Burk et al. (2005) found that instead of dimers, CAR monomers interacted with P-gp gene. The group suggested that this could separate CAR mediated regulation of P-gp and CYP3A4 from each other. Substrate selection of PXR and CAR is also partially overlapping (Moore et al. 2002). There was however much fewer compounds that activated CAR receptors which means that inductions is more likely mediated by PXR rather than CAR.

5 P-GLYCOPROTEIN LIGANDS

5.1 Recognizing molecules interacting with P-glycoprotein

Some molecules interact with P-gp and are transported (substrates), some inhibit the function P-gp (inhibitors), and some act as both inhibitors and substrates. In addition there are inducers which improve the P-gp function. P-gp ligands can be studied with several *in vivo* and *in vitro* assays (Didziapetris et al. 2003). In *in vivo* assays utilize P-gp transgenic and P-gp gene knockout rodents such as mice and rats. *In vitro* assays

include transport assays, membrane accumulation assays and ATPase assay. In addition cancer lines are used to study the ability of study compounds to increase the effect of cytotoxic agents (Zamora et al. 1988). The ATPase assay is based on the fact that P-gp efflux is dependent on ATP hydrolysis thus the ATPase activity of the test drug is measured (Sarkadi et al. 1992). Cell based assays use cell lines that express P-gp (such as Caco-2) and P-gp gene transfected cell lines (such as MDCK and Chinese hamster ovary cell line). The membrane accumulation method utilizes human P-gp transfected membrane (Aanismaa 2007). MDCKII-MDR1 transport cell assay and P-gp membrane accumulation assay are used and explained more in detail in experimental part. More direct study methods are functional analysis with the help of photo-affinity labeling or direct binding studies with thiol cross-linking agents (Bruggeman et al. 1992, Ecker et al. 2002). These methods have been utilized especially when the binding sites of the protein have been studied.

The study methods are mostly based on interaction between recognized substrates or modulators. FDA offers a list of acceptable P-gp substrates and inhibitors for *in vitro* studies (Table 2). One problem, however is that some compounds can act as both a substrate and an inhibitor (such as verapamil) which complicate interpretation of the results (Didziapetris et al. 2003, Srivalli and Lakshmi 2012). Also many molecules are substrates or modulators of more than one transporter (such as cyclosporine A) (Rebello et al. 2011).

Table 2 Acceptable P-gp substrates and inhibitors for *in vitro* studies according to FDA (2012)

***In vitro* substrates**	***In vitro* inhibitors**
digoxin	cyclosporine A
loperamide	elacridar (GF120918, GG918)
quinidine	ketoconazole
talinolol	LY335979
vinblastine	nelfinavir
	quinidine
	reserpine
	ritonavir

saquinavir
tacrolimus
valspodar (PSC833)
verapamil

Computational methods are used extensively for substrate screening. The substrates can be recognized by docking experiments where different molecules are fitted in to the binding pocket of the protein to find out which molecules most likely. Alternatively, substrate screening can be based on known ligands and their structure (Matsson and Artursson 2013).

Now that the crystal structure of mouse P-gp is known it can be utilized to predict structure of human P-gp (Chen et al. 2012). *In silico* quantitative structure-activity relationship (QSAR) assay have been used in recognizing P-gp ligands with variable results (Sharom et al. 2005). Often *in silico* methods are based on the experimental data set result which defines the P-gp substrates and modulators. The largest dataset included 1273 molecules from which 797 were P-gp inhibitors (Chen et al. 2011). The data sets however, are a result of variable study methods, laboratories and definers of the affinity, thus making incisive predictions based on them is challenging (Stouch and Gudmundsson 2002).

P-gp ligand specificity is affected by many factors such as molecular weight (MW), logP, number of aromatic groups, number and strength of hydrogen accepting groups (~number of O and N atoms), acidity, surface area, and 2/3D pharmacophore (Didziapetris et al. 2003). Some factors such as logP and hydrophobicity seem to be more important to inhibitors than to substrates but finding distinctive features between substrates and modulators is very challenging.

5.2 Is there a typical P-glycoprotein interacting molecule?

5.2.1 Physicochemical properties of drug and rule of four

Didziapetris et al. (2003) studied P-gp substrate specificity with a stepwise classification structure–activity relationship (C-SAR) method using 1000 compounds. The group used compound size, hydrogen bond accepting ability (eg. sum of N and O atoms) and ionization as defining factors. They found that molecules follow loosely a "rule of fours" (related to Lipinski's rule of five) thus the compounds which have sum of N and O atoms less than 8, molecular mass more than 400 and acidic pKa less than 4 are most likely P-gp substrates. The group noted that a basic compound with large MW and high H-bond accepting is the most probable substrate.

The optimal size of less than 400 Da defined for P-gp substrates by the "rule of four" is controversy because according to the common view P-gp substrates have wide dispersion in molecular weights the rule of fours (Srivalli and Lakshmi 2012). On the other hand, "rule of fours" is in agreement with previous studies about the importance of N- and O-atoms. Need for the presence of nitrogen atom has been mentioned in several other studies and some studies suggests that the sum of the O and N-atoms is important like "rule of fours" states (Tang-Wai et al. 1993, Klopman et al. 1997, Pajeva and Wiese 2002, Cianchetta et al. 2005).

According to "rule of fours" hydrophobicity and number of aromatic groups do not influence on substrate specificity. This is in agreement with the results El Ela and coworkers (2004) obtained when they studied structure activity of 15 psychoactive compounds. With psychoactive compounds logP ranging from 1.56 to 4.5 there was no correlation between logP and affinity to P-gp. However in one previous study it was demonstrated that molecule with logP higher than -1 can be P-gp inhibitor (Dellinger et al. 1992). This could be reason why there was no difference seen in the study of el Ela et al. (2004) with rather hydrophilic compound group. Also number of studied compounds was very low which is why the results can not be generalized.

According to the nature of binding cavity the most likely binding force between substrate and cavity is π-π binding thus the lipophilicity would be expected to affect the binding affinity (Lugo and Sharom 2005). Klopman et al. (1997) listed logP as an important factor for inhibitory effect. Also in another early study lipid soluble (logP ≥ 1) compounds in physiological pH were able to increase vinca-alcaloid cytotoxicity while almost all of the water soluble compounds were not (Zamora et al. 1988). This suggests that in order to compete from binding to P-gp with vinca-alcaloids the molecule needs to be lipophilic. However also in the mentioned study the number of compounds was low (about 20). Therefore according to studies made so far the lipophilicity of P-gp interacting molecule seems to be important in some compound groups but it can not be said to be discriminatory factor.

5.2.2 Spatial structure and hydrogen bonding

The riddle has been approached by investigating the body structure of ligands. Several studies show that the position of hydrogen bonding groups is an important descriptive factor for P-gp affinity (Seelig 1998, Seelig and Landwojtowicz 2000, Didziapetris et al. 2003, Pajeva and Wiese 2002, Cianchetta et al. 2005). Seelig (1998) proposed that P-gp substrate has two (type I substrate) or three (type II substrate) electron donor groups which have designated separation between each other. Type I pharmacophore has 2.5 ± 0.3 Å separation between two electron donor groups and type II has 4.6 ± 0.6 Å separation between two outer electron donor groups or alternatively 2.5 ± 0.3 Å separation between all three groups. Based on testing of 100 compounds it was concluded that when the number and "strength" of hydrogen bonding groups increased the probability of drug being a P-gp substrate or inducer increased. Also Pajeva and Wiese (2002) found that increasing number of wide spread hydrophobic groups are important for P-gp interacting compound.

Type I and II molecules were further tested by determining the dependency of transport rate with air-water coefficient (K_{aw}) and critical micelle concentration (CMC) (Seelig and Landwojtowicz 2000). K_{aw} and K_m (Michaelis-menten constant) were linearly dependent and the higher the amount and strength of hydrogen bond donors (HD) in the

compound structure the lower was the transport rate (V_{max}) obtained from ATPase assay. The group proposed that hydrogen bond formation helps ligands to interact with P-gp but because of them the partitioning to the lipid membrane is the rate limiting step which would explain the lower transport rate.

Cianchetta et al. (2005) demonstrated that from two defining factors, GRIND (Grid independent descriptors) pharmacophore and physicochemical properties, the first one had clearly higher correlation with inhibitory potency. This suggests that the core spatial structure is more important descriptor than simply physicochemical properties. Inhibitory effect of 129 compounds was first studied with calcein-AM assay and then a 3D QSAR analysis was performed. Based on their findings they suggested that the ligand recognition happens by two hydrophobic and two hydrogen-bond acceptor groups which have 16.5 Å and 11.5 Å separations, respectively.

Recently it was demonstrated that combination of rigid and flexible groups in ligand structure causes good binding affinity (Orlandi et al. 2012). N,N-bis(cyclohexanol) amine scaffold gives this kind of structure to a series of newly developed P-gp inhibitors thus the group suggested that this scaffold structure can be exploited in designing of P-gp regulators (Martelli et al. 2009). The structure of these new compounds is different from previous P-gp inhibitors in many ways but they have several hydrogen bonding groups. The base structure of the compounds resembles the “ideal” structure Klopman and coworkers suggested already in 1997 for anti-cancer drugs based on the basic structure of paclitaxel. Ekins et al. (2002) produced a 3D pharmacophore for P-gp inhibitor based on 16 verapamil metabolites which have inhibitory effect on P-gp (Figure 13). Important features in the structure were one aromatic ring, two hydrophobic areas, hydrogen bond accepting group and wide spread form.

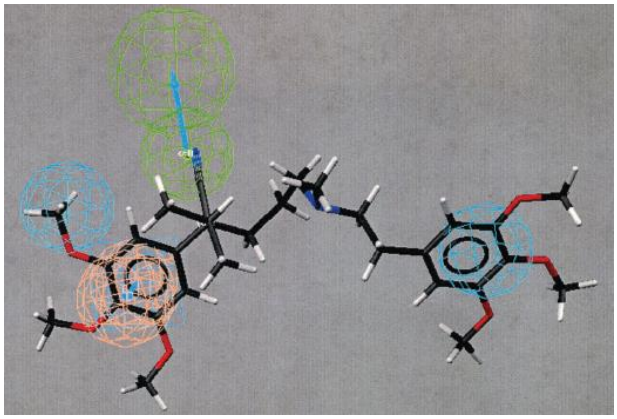


Figure 13. Pharmacophore (colored balls, green for hydrogen bond acceptor, orange for aromatic area and cyan for hydrophobic area) for P-gp inhibitor created in based on verapamil metabolites and molecule Lu-47474 (black, white and red lines) fitted to the pharmacophore (obtained from Ekins et al. 2002)

5.3 Therapeutic groups

Most commonly P-gp substrate or modulator belongs to the therapeutic group of anticancer and cytotoxic drugs (Gottesman and Pastan 1993, Ambudcar et al. 1999, Linardi et al. 2006, Hennessy and Spears 2007). There are several P-gp interacting drugs also among immunosuppressive drugs, steroids, HIV protease inhibitors, antimicrobial, and cardiac drugs and in many more groups. Examples of P-gp ligands from different therapeutic groups are presented in Table 3. Also lipids, several fruits herbs and natural product constituents can interact with P-gp (Srivalli and Lakshmi 2012). In the experimental part P-gp mediated transport of β-adrenoceptor antagonists celiprolol and talinolol, H1 histamine receptor antagonists fexofenadine and cardiac drug aliskiren are investigated.

Table 3. Examples of P-gp substrates and inhibitors from different therapeutic groups (Adapted from Linardi et al. 2006 and Srivalli and Lakshmi 2012)

Substrates	
Antiacids	Cimetidine, Ranitide
Antibiotics	Erytromycin, Tetracycline, Rifampicin, Levofloxacin
Antiemetic	Ondansetron
Antitumor agents	Paclitaxel, Doxorubicin, Daunorubicin, Vinblastine, Vincristine,

	Actinomycin D, Docetaxel, Etoposide, Imatinib
β-Adrenoceptor antagonists	Bunitrolol, Carvedilol, Celiprolol, Talinolol, Reserpine
Ca^{++} Channel Blockers	Diltiazem, Mibefradil
Cardiac drugs/Antiarrhytmics	Digitoxin, Digoxin
H1 histamine receptor antagonists	Fexofenadine, Terfenadine
HIV-protease Inhibitors	Ampenavir, Indinavir, Nelfinavir, Saquinavir, Ritonavir
Immunosupressants	Cyclosporine A, Sirolimus, Tacrolimus
Opioids	Loperamide, Domperidone, Morphine, Pentazocine, Methadone, Asimadoline, Fentanyl
Steroids	Dexametasone, Methylprednisolone, Aldosterone, Progesterone, Hydrocortisone, Cortisol, Corticosterone
Others	Colchicine, Itraconazole, Phenothiazines, Ivermectin
Inhibitors	
Antiarrhythmics	Amiodarone, Quinidine, Verapamil, Felodipine, Nifidipine, Dilitiazem
Anticancer drugs	Actinomycin D, Doxorubicin, Vinblastine
Antibiotics	Clarithromycin, Erythromycin.
Antidepressants	Paroxetine, Sertraline, Desmethylsertraline
Proton pump inhibitors	Esomeprazole, Lansoprazole, Omeprazole, Pantoprazole
Others	Valspodar (PSC), Cyclosporine A, Ketoconazole

II EXPERIMENTAL WORK

6 BACKGROUND

Over the past two-three decades the involvement of transporters in drug distribution, metabolism and excretion has come to our knowledge. *In vitro* studies are used to screen transporter substrates and inhibitors to predict drug-drug interactions. Replacing *in vivo* studies with *in vitro* studies saves money and time and is more ethical. However there are still many uncertain aspects that need to be considered and more clear guidelines are needed for *in vitro* transporter studies (Giacomini et al. 2010). In this experimental part four P-gp substrates are analyzed with two *in vitro* methods and the results are reviewed in light of previous *in vitro* and *in vivo* studies.

6.1 *In vitro* study methods

Both FDA and EMA recommend using Caco-2 cells for investigating transporter substrates (EMA 2012, FDA 2012). An alternative tool for research is a cell line that overexpresses a human transporter protein such as MDCKII (FDA 2012). MDCKII (Madin Darby Canine Kidney) -cell line is originally renal epithelia from dog (Tang et al. 2002). The cell line has been transfected with human P-glycoprotein (P-gp) coding gene ABCB1 to produce MDCKII-MDR1-cell line. Tang et al. (2002) demonstrated that MDCKII-MDR1 cell line expresses higher levels of P-gp than MCDKII wild type (MDCKII-wt) or caco-2 cells. MDCKII cells form uniform cell monolayer and tight junctions substantially faster than caco-2 cells (3 days compared to 30 days) (Irvine et al. 1999).

Another option (though not approved by authorities) is to use membrane vesicles which are made from cell membrane and inverted into inside-out conformation (Giacomini et al. 2010). The cytoplasmic membrane and thus also the binding site for efflux transporters is facing outside the vesicle (Glavinas et al. 2008). The inside-out vesicles

were first prepared and characterized from red blood cell plasma ghost membranes (Steck et al. 1970). Vesicular transport assay has been utilized already in 1988 for studying P-gp mediated transport (Horio et al. 1988). The vesicles were prepared from multidrug resistant human carcinoma cells containing p-glycoprotein and the group was able to demonstrate ATP-dependent transport of vinblastine. Membrane vesicles prepared from Sf9 (*Spodoptera frugiperda* ovarian) cells expressing P-gp were first used to demonstrate that a drug can stimulate ATPase-activity (Sarkadi et al. 1992). Liberation of inorganic phosphate (Pi) was higher when a drug and Mg-ATP was incubated with vesicles infected with MDR1-baculovirus than uninfected vesicles. The advantage in using the Sf9 membrane vesicles is the lack of human transporter expression because the cells are insect-origin. Common limitation in vesicular assay is the lack of high permeability drugs showing difference between passive and ATP-dependent transport (Glavinas et al. 2008).

6.2 Properties of the selected test drugs

A renin-enzyme inhibitor aliskiren (Rasilez®, Tekturna®) is used for treatment of essential hypertension alone or with other blood pressure lowering agents (drug@FDA, www.ema.europa.eu). An adrenergic β1-receptor antagonist and partial β2 receptor agonist celiprolol (Selectol®, Celectol®, Cardem®) and a selective β1 receptor talinolol (Cordanum®) are used for treatment of high blood pressure and coronary decease (Duodecim 2013, Martindale 2007). A selective H1-receptor antagonist fexofenadine (brand names Telfast® and Allegra®) is used to treat seasonal allergic rhinitis and chronic idiopathic urticarial. (drug@FDA). A P-gp inhibitor itraconazole (brand name Sporanox®) is a synthetic triazole antifungal drug which is used for treatment of lung and general fungal infections and nail fungal infections (drugs@FDA).

The fact that mentioned drugs are used to treat long-term conditions means that they are probably used simultaneously with other medical agents. This increases the risk for drug-drug interaction which can potentially cause loss of effect or undesirable side effects by altering the ADME (absorption, distribution, metabolism and elimination) properties of the drug(s). Aliskiren, fexofenadine, talinolol and celiprolol show P-gp

dependent kinetics *in vivo*. The first three drugs are classified as P-gp substrates in FDA drug interaction studies-guidance for industry (FDA 2012). P-gp mediated interactions of these drugs have been demonstrated to be clinically significant (Table 4).

Table 4. *In vivo* interactions of the test drugs and classification of interaction according to SFINX-PHARAO (Class C3 indicates clinically significant interaction which can tolerated with dose adjustment, D3 indicates clinically significant interaction which should be avoided). Arrow up means increasing and arrow down decreasing parameter when P-gp substrate is administered with inhibitor/inducer compared to the situation when drug is administered alone.

		***In vivo* difference**		
Substrate	**Inhibitor/inducer**	**AUC**	**C_{max}**	**SFINX-significance class[10]**
Fexofenadine	Itraconazole[1]	↑ 2-3,3x	↑ 1.8-2.3x	C3 (P-gp)
	Carbamazepine[2]	0.4-0.6x	↓ 0.7 x	C3 (P-gp)
	Verapamil[3]	1.7-2.1x	↑ 1.5-2.2x	C3 (P-gp)
Celiprolol	Itraconazole[4]	1,8x	↑ 1,3x	C3 (P-gp/CYP3A4)
	Rifampicin[5]	0.4x	↓ 0.7x	C3 (P-gp)
Talinolol	Rifampicin[6]	0.6	↓ 0.6x	-
	Carbamazepine[7]	0.85x	-	-
Aliskiren	Itraconazole[8]	6x	-	D3 (P-gp)
	Verapamil[9]	↑ 2x	↑ 2x	C3 (P-gp)

*SFINX (Swedish, Finnish, Interaction X-referencing) drug interaction database
[1] Shimizu et al. 2006a, Shimizu et al. 2006b, Tateishi et al.2008
[2] Akamine et al. 2012, Yamada et al. 2009
[3] Lemma et al. 2006, Sakugawa et al. 2009
[4] Lilja et al. 2003
[5] Lilja et al. 2004a
[6] Westphal et al. 2000
[7] Giessmann et al. 2004
[8] Tapaninen et al. 2011
[9] Rebollo et al. 2011
[10] Duodecim 2013

Pharmacokinetic properties of the test drugs are presented in (Table 5). All four drugs have low bioavailability which could refer to low solubility or permeability, gut wall metabolism or efflux transport, possible mediated by P-gp. The drugs are excreted mainly with feces or via urine (Lilja et al. 2004a, Kagan et al. 2007, Waldmeir et al.

2007, Lappin et al. 2010). Only 1-5 % of the absorbed drug dose is metabolized in the body which makes the drugs interesting P-gp probe drugs because expression of metabolizing enzymes doesn't affect their pharmacokinetics (Lilja et al. 2004a, Kagan et al. 2007, Martindale 2007, Waldmeir et al. 2007). The dose dependency rather than time dependency of talinolol pharmacokinetics also suggests that there is some active, saturable mechanism involved (Kagan et al. 2010). In addition celiprolol and fexofenadine have shown saturable pharmacokinetics and dose dependent bioavailability (Karlsson et al. 1993, Lappin et al 2010).

Table 5. Pharmacokinetic properties of test drugs

Parameter	Unit	Aliskiren [1]	Celiprolol [3]	Fexofenadine [5]	Talinolol [7]
Dose	mg	150	100	60	50
T_{max}	h	1 (0-5)	4 (3-6)	2 (0.5–4)	3.3 ± 1.5
C_{max}	ng/ml	142 ± 42	277 ± 229	332±252	45 ± 24
$AUC_{0-\infty}$	ng h/ml	625 ± 293	1266 ± 791	1801 ± 979	427 ± 192
T ½ (p.o.)	h	34.0 ± 9.7	4.8 ± 1.3	6.6 ±3.1	9.1 ± 4.1
BA**	%	2,5 [2]	30-70 [4]	30 [6]	55 [8]
CL renal	ml/min	24.67 ± 4	205 ± 45.5	55.6 ± 21.8	220 ± 102
Ae *	mg	0.567 ± 0.289	13.9 ± 6.5	5.6 ± 3.3	4.5 ±1.9

*Ae = Amount excreted to urine
**Bioavailability
[1] Tapaninen et al. 2011. Ae 12 hours.
[2] drugs@FDA
[3]Lilja et al. 2003
[4] Lilja et al. 2004a
[5] Tateishi et al. 2008 Ae 0-24 hours
[6] Lappin et al. 2010. Fexofenadine dose 120 mg
[7] Schwartz et al. 2007 Urinary excretion of unchanged talinolol from 0 to 48 h. CL_r from 0 to 24 h.
[8] Kagan et al. 200. Dose 100 mg

The molecular formulas and physicochemical properties of the drugs are presented in Table 7. and Table 8. The aqueous and lipid solubility of the drugs is variable. According to FDA a drug substance is highly soluble if the ratio of highest oral dose (mg) to the solubility (mg/ml) is less than 250 ml over the pH range 1-7.5 (at 37 ˚C). Aliskiren, celiprolol and talinolol meet these criteria at pH 7.4 but fexofenadine is not highly soluble according to this definition (Table 6). The $LogD_{7.4}$ values for the test

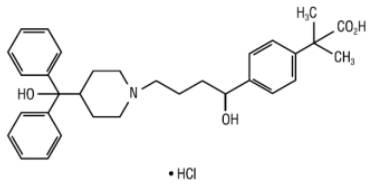
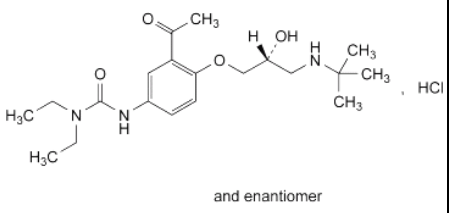
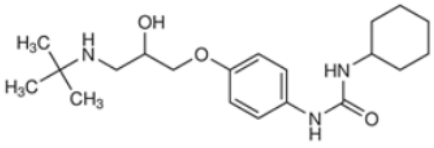
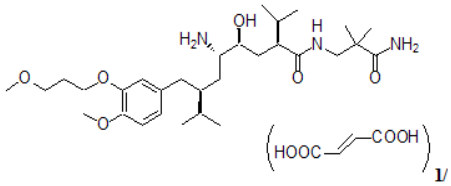
drugs vary from -0.22 to 2.30. Fexofenadine which is the most hydrophilic compound in this group is classified as a low permeable drug and has rather low permeability through Caco-2 cells (Chen 2007).

Table 6. Solubility of the maximal oral daily dose of the test drugs in 250 ml (at pH 7.4 and 37 ˚C)

	Aliskiren	**Celiprolol**	**Fexofenadine**	**Talinolol**
Maximal oral daily dose (mg)	300 [1]	600 [1]	180 [1]	300 [2]
Solubility in 250 ml (mg)	>300	1830	2.5	563

[1] Micromedex 2.0
[2] Martindale 2007

Table 7. Molecular formulas for investigatory-drugs

Fexofenadine (as hydrochloride salt)[1]	**Celiprolol** (as hydrochloride salt)[2]
	
Talinolol[3]	**Aliskiren** (as hemifurete salt)[1]
	

[1] drugs@FDA
[2] European Pharmacopea 7th edition
[3]Martindale 2007
[3]European Bioinformatics Institute 2013

Table 8. Physicochemical properties of the P-gp substrates

Parameter	**Aliskiren**	**Celiprolol**	**Fexofenadine**	**Talinolol**
Molecular mass (g/mol)	551.8[3]	379.49[1]	501.66 [2]	363,49[1]

Aqueous solubility (pH 7.4) (mg/ml)	>350[3]	7.32[1]	0.81[6]	2.26[1]
P_{app} (cm/s x10^{-6})	-	-	0.17 ± 0.08[4]	1.08 ±0.29[5]
logP[1]	2.74	1.92	4.8	3.2
$logD_{7.4}$[1]	0.07	-0.22	2.30	1.03
$logD_{5.5}$[1]	-0.35	-1.14	2.28	0.14
pKa[1]	9.49 (base)	9.11 (base)	9.56 (acid) 4.43 (base)	0.89 (base)
PSA[1]	146.1	90.9	81	82.62

[1] Calculated by ACD labs 8.08 (Canada)
[2] European pharmacopeia 7th edition
[3] drugs@FDA
[4] Petri et al. 2004 Determined in caco-2 cells (A-B) with 50 µM concentration
[5] Deferme et al. 2002 Determined in caco-2 cells (A-B) with 100 µM concentration
[6] Chen 2007

7 AIMS OF THE STUDY

The purpose of this experimental work was to study P-gp-mediated transport of aliskiren, celiprolol, fexofenadine and talinolol in MDCKII-MDR1-cells and in membrane vesicles extracted from P-gp expressing Sf9 cells. The aims of the study were

(1) to assess the feasibility of using the vesicle assay in P-gp interaction studies with hydro- and lipophilic drugs,

(2) to determine K_m and V_{max}–values for aliskiren with vesicles and cells in order to compare the methods,

(4) to determine efflux ratios for the substrates with and without inhibitors in order to find out if they are *in vitro* P-gp substrates and

(5) to compare *in vitro* results to *in vivo* results in order to see if they can be used to predict *in vivo* effect.

8 MATERIALS AND METHODS

8.1 Drug compounds

Celiprolol hydrochloride, fexofenadine, talinolol and itraconazole were obtained from Santa Cruz Biotechnology (Germany). Aliskiren hydrochloride was obtained from Santa Cruz Biotechnology (Germany) for vesicular transport assay and from Toronto Research Chemicals Inc. (Germany) for the cell permeability test.

For the vecicular transport assay stock solutions of 10 mM were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO in test solution was 1 %. For the cell permeability experiments fexofenadine and talinolol stock solution of 10 mM and aliskiren hydrochloride stock solution of 5mM were prepared in DMSO. Celiprolol hydrochloride stock solution was prepared in Milliq water. Test solutions were prepared in transport buffer. The final concentration of DMSO in the test solution was 0.1-0.2 % except for the test for K_m-determination of aliskiren where the DMSO concentration was 0.6 %. Itraconazole 10 mM stock solution was prepared in DMSO and further diluted to 10 µM test solution in transport buffer.

8.2 Control compounds

Control molecules Lucifer yellow and paracetamol (one experiment) were used in cell assay. Cyclosporine was used to study passive permeation of P-gp substrates. Paracetamol was obtained from Santa Cruz Biotechnology (Germany) and cyclosporine and Lucifer yellow were obtained from Sigma-Aldrich (Germany).

Lucifer yellow is frequently used as a paracellular marker. It is highly fluorescent and therefore can be measured optically. It was used to control the integrity and uniform quality of the cell monolayer. Lucifer yellow 20 mM stock solution was prepared in transport buffer and further diluted to 250 µM test solution. One part of the experimental work was to test the effect of polar organic solvent DMSO on cell

integrity. 0.1-1 % (v/v) of DMSO was added to the 250 µM Lucifer yellow test solution to produce DMSO concentration of 0.1-5 % (v/v).

An analgetic and antipyretic agent paracetamol (acetaminophen) was used as a model compound for a high or low permeability boundary drug. Paracetamol stock solution of 10 mM and test solution of 50 µM were prepared in transporter buffer. Cyclosporine is an inhibitor of several transporters (P-gp, OATPs, BCRP) and therefore it can used to prevent active transport and study the passive permeability (FDA 2012). Cyclosporine concentration was used in the cell assay to find out the passive permeability of the test drugs. Cyclosporine 10 mM stock solutions were prepared in DMSO and further diluted to 10 µM test solution in transport buffer.

8.3 Vesicular transport assay

The membrane vesicles were prepared from P-gp-gene containing recombinant baculovirus -infected Sf9 (*Spodoptera frugiperda*) insect cells. The total protein concentration of stock solution was 5 mg/ml. The membranes were supplemented with cholesterol to improve function of P-gp and gain cholesterol concentration closer to mammal cells. The cholesterol concentration was about 80 µg/ mg per total protein concentration as measured with the Amplex Red Cholesterol Assay kit (Invitrogen, USA).

8.3.1 The test protocol

The membrane vesicle solution with protein concentration of 5 mg/ml and cholesterol concentration of 5 mM was diluted 1:5 in freshly prepared assay mix (5 ml 0.1 M MOPS-Tris, 5 ml 0.14 M KCl and 0.75 ml 0.1 M $MgCl_2$). 50 µl of the membrane vesicle solution per well was distributed to 96-well plate. The P-gp substrate was added either directly to the membrane solution before pipetting into to the wells, or in 0.75 µl to the wells. The plate, freshly prepared 12 mM Mg-adenosine triphosphate solution (Mg-ATP) in assay mix and plain assay mix were incubated at 37 C˚ for 10 minutes.

The reaction was initiated by adding 25 µl of Mg-ATP-solution or assay mix to desired wells.

The plate was incubated in 37 C° for predetermined time and the reaction was stopped by adding 200 µl of ice cold washing mix (200 ml 0.1 M MOPS-Tris, 35 ml 1 M KCl and 265 ml MilliQ water) to the wells. The samples were transferred to the 96-well filter plate and the solution was removed by suction. The wells were washed five times with ice cold washing mix and after that the plate was allowed to dry in room temperature for 60 minutes. The vesicles were broken by incubating the wells with 100 µl of 0.1 M ammonium hydroxide solution and the samples were transferred to 96-well plate by suction.

Transported drug amount inside the vesicles was determined with and without ATP in triplicates. ATP dependent transport (molecules/time/mg of protein) was calculated by reducing transport rate in well incubated without ATP from transport rate in wells incubated with ATP.

8.3.2 Test circumstances and concentrations

At first the vesicular transport assay was performed for all the test drugs with concentrations of 1, 10 and 100 µM and incubation time 30 min in order to determine the best concentration for incubation time optimizing. The concentration that gave the biggest ratio between active and passive transport was chosen for further analysis. The incubation time was optimized by incubating test plates for 1, 3, 10 or 30 min. Celiprolol concentrations of 1, 2, 5, 10, 20, 50, 75 and 100 µM and aliskiren concentrations of 0.5, 1, 2, 4, 8, 10, 15 and 30 µM were used for the determination of K_m-values.

8.4 Cell permeability assay

8.4.1 Cell culture of MDCKII-MDR1-cells

The Madin-Darby canine kidney II (MDCKII) cells expressing human multidrug resistance protein I (MDR1) (P-glycoprotein) were originally obtained from Netherland Cancer Institute (Amsterdam). The cells were grown in cell culture flasks and split twice a week. The growth medium was prepared from D-MEM (Dulbecco's Modified Eagle's medium) (31885, Gibco USA) which was supplemented with 10 % FBS (foetal bovine serum) and Penicillin-Streptomycin solution (1 %, Gibco). Before splitting the cells were washed with DPBS (Dulbecco's phosphate buffered saline) (-Ca and -Mg, 14200 Gibco). The cells were detached from the bottom of the flask by incubating them with TrypLe Express (12604 Gibco) for 8-10 min and divided in 1:9.

The cells were grown on 12-well plate with polyester membrane inserts (3460, Costar Transwell USA) with diameter of 0.4μm and area 1.12 cm^2 (Figure 14). The cell density was measured with the help of Bürker chamber and microscope and seeding density was adjusted to 0.8 x 10^6 cells /ml. Before permeability experiments the cells were grown at 37 C° and 5 % CO_2 for 5 days. Media was changed every day in both apical and basolateral side. The passage number of the cells used in the experiments with talinolol, celiprolol, aliskiren, fexofenadine and paracetamol varied between 13 and 17 and with Lucifer yellow between 12 and 18.

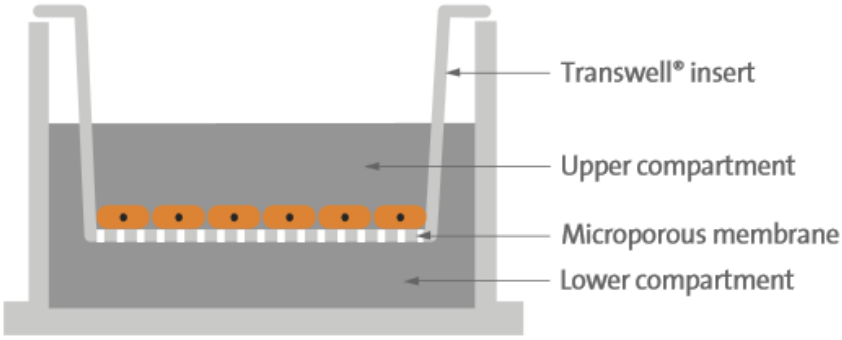


Figure 14. Design of the Transwell permeable supports. The upper and the lower compartment represents the apical and the basolateral compartments, respectively. The cells grow on the microporous membrane (Corning Life Sciencis 2013).

8.4.2 Determination of trans-epithelial electrical resistance

The integrity of the cell monolayer was evaluated by measuring trans-epithelial electrical resistance (TEER, Ωcm^2). Values were determined with EVOM (Epithelial Voltohmmeter) with STX2 electrode (World Precision Instruments, USA). TEER values were measured at 37 C° in growth media at 4 and 5 days after the seeding and in buffer before and after the experiment. Before measuring TEER-values in buffer the monolayers were washed 2 times with pre-warmed transport buffer solution. The TEER-values of the epithelial monolayer were calculated with Equation 1.

Equation 1

$$TEER\ (cells) = TEER\ (filter\ with\ cells) - TEER\ (filter\ with\ no\ cells)$$

8.4.3 Determination of permeability of the P-gp substrates

Celiprolol, fexofenadine and talinolol concentrations of 10 µM and aliskiren concentration of 2 µM were used for determination of permeability. The permeability tests for P-gp substrates were performed from the apical to the basolateral side (A-B) and from the basolateral to the apical side (B-A). In addition, permeability of fexofenadine (10 µM), celiprolol (10 µM) and aliskiren (2 µM) was determined with 10

µM itraconazole for both directions. Permeability of aliskiren (2 µM) and talinolol (10 µM) with cyclosporine was determined from A to B and for celiprolol (10 µM) in both directions. The K_m-determination of aliskiren was performed from the apical to the basolateral side and aliskiren concentrations of 0.5, 1, 2, 4, 6, 12, 30, 100 µM and was used for determination of K_m and V_{max} values.

Experiments were performed in transport buffer: HBSS (Hank's balanced salt solution 14025, Gibco) supplemented with 10 mM HEPES (15630 Gibco) (pH 7.4). The liquid volume was 1500 µl and 500 µl at the basolateral and the apical side, respectively. Before experiments the cell monolayers were washed twice with the transport buffer and incubated for 30 min at 37 C˚. Test was performed in 12-well plates (3512, Corning costar USA) which were pre-incubated for 10 min at 37 C˚ with test solution (B-A test) or buffer solution (A-B). At the beginning of the experiment the inserts containing the cell monolayer were emptied of buffer and transferred to the test plate. The test solution or the transport buffer was added to the apical side with 60 s interval. Experiments were performed at 37 C˚ and shaking 200 rpm. Samples of 200 µl were taken from receiver side at four time points and were replaced with warm transport buffer. Samples of test solutions were taken at the beginning and at the end of the experiment.

Apparent permeability ($P_{app,}$ cm/s) was calculated with Equation 2 where dM/dt (µmol/s) is the slope of the drug concentration in the receiver chamber with time, C0 (µM) is the concentration in the donor chamber when t=0 and A (cm^2) is the filter surface area.

Equation 2

$$Papp = \frac{dM}{dt} \times \frac{1}{(A \times C0)}$$

The efflux ratio (ER) was calculated for test drugs with and without inhibitors with Equation 3 where P_{app} (A to B) is the apparent permeability of the substrate into the basolateral (absorptive) direction and P_{app} (B to A) is the apparent permeability of the substrate to the apical (secretive) direction.

Equation 3

$$ER = \frac{Papp(B\ to\ A)}{Papp\ (A\ to\ B)}$$

8.5 Analytics

The samples were frozen at -20 ˚C and stored for the analysis (variable time). Lucifer yellow was measured within an hour by Fluorometry Varioscan (Thermo Electron corporation, Massachusetts, USA) in a black 96-well plate with clear bottom (3631, Corning costar, USA). The excitation and emission wavelengths of 425 and 538 nm were used, respectively.

The other compounds were analyzed with combined ultra-performance liquid chromatorgraphy and mass spectrometry (UPLC-MS). The liquid chromatography separation of samples was performed with Waters Acquity UPLC (Waters, Massachusetts, USA) (HSS T3 column, column dimensions 2.1 x 100 mm and particle size 1.8 µm, flow rate 0.3 ml/min, injection volume 0.5 µl, T 25 °C, mobile phases H2O + 0.1 % Formic Acid (gradient 90-10-90 %) and acetonitrile + 0.1 % Formic Acid (gradient 10-95-10 %)). The mass spectrometric measurements were performed with Waters TQ-S triple quadrupole mass spectrometer (Waters, Massachusetts, USA) (electrospray ionization: ESI+, capillary voltage 3.7 kV, desolvation temperature 500 °C and gas flow 800 l/h, cone gas flow 150 l/h, desolvation, collision and cone gas nitrogen, scan mode MRM, dwell time 0.034-0.068 s and retention time 1.8-2.2 min).

9 RESULTS

9.1 Vesicular transport assay

9.1.1 ATP dependent transport with different test concentrations

The ATP dependent transport of talinolol, fexofenadine and celiprolol with test concentrations 1, 10 and 100 µM is presented in Table 9. The concentration optimization for aliskiren was not performed because this was done earlier for the used vesicles by Koskenkorva (2012). Talinolol and celiprolol failed to show ATP dependent transport with concentration 10 and 100 µM and fexofenadine failed show active transport with concentration 100 µM. The ATP-dependent transport was highest with concentration 1µM for fexofenadine. Also highest ratio between +ATP/-ATP-transport was measured for fexofenadine and lowest for celiprolol.

Table 9. ATP-dependent transport of talinolol, fexofenadine and celiprolol with concentrations 1, 10, and 100 µM (n=3) in MDR1-Sf9 membrane vesicle assay. Incubation time was 30 min. (Average ± SD)

Concentration (µM)	1	10	100
Talinolol			
ATP-dependent transport (pmol/min/mg protein)	0.168 ± 0.1	-	-
Ratio (+ATP/-ATP)	2.1 ± 1.2	0.73 ± 0.49	0.66 ± 0.43
Fexofenadine			
ATP-dependent transport (pmol/min/mg protein)	0.348 ± 0.34	0.215 ± 0.78	-
Ratio (+ATP/-ATP)	2.6 ± 1.7	1.3 ± 1.2	0.55 ± 0.82
Celiprolol			
ATP-dependent transport (pmol/min/mg protein)	0.044 ± 0.052	-	-
Ratio (+ATP/-ATP)	1.6 ± 0.99	0.46 ± 0.79	-

9.1.2 ATP dependent transport with different incubation times

The ATP dependent transport of talinolol, fexofenadine and aliskiren (1 µM) with test incubation times 1, 3, 10 and 30 min is presented in Table 10. The incubation time optimization failed with celiprolol and further studies were performed with incubation time 1 min. The transport rate decreased when the incubation time increased with all three compounds. Talinolol failed to show active transport with incubation time 30 min.

Table 10. ATP-dependent transport of talinolol, fexofenadine and aliskiren with incubation times 1, 3, 10, and 30 min (n=3) in sf9 membrane vesicle assay. 1µM concentration was used (Average ± SD)

Incubation time (min)	1	3	10	30
Talinolol				
ATP-dependent transport (pmol/min/mg protein)	4.7 ± 7.3	0.56 ± 0.3	0.22 ± 0.0	-
Ratio (+ATP/-ATP)	2.5 ± 2.3	1.2 ± 0.28	1.1 ± 0.11	1.0 ± 0.33
Fexofenadine				
ATP-dependent transport (pmol/min/mg protein)	2.4 ± 1.4	0.37 ± 0.24	0.11 ± 0.13	0.04 ± 0.02
Ratio (+ATP/-ATP)	2.2 ± 0.79	1.4 ± 0.32	1.3 ± 0.42	1.4 ± 0.27
Aliskiren				
ATP-dependent transport (pmol/min/mg protein)	14 ± 6.7	5.4 ± 2.2	1.6 ± 0.8	0.76 ± 0.31
Ratio (+ATP/-ATP)	1.6 ± 0.37	1.9 ± 0.4	2.1 ± 0.7	2.9 ± 1.2

9.1.3 K_m determination

The results from K_m determination of celiprolol are presented in Figure 15. ATP dependent transport of celiprolol did not saturate with concentrations 1-100 µM and transport rate increased with increasing concentration. The maximal transport rate of celiprolol with concentration of 100 µM was 170 ± 144 pmol/mg/min.

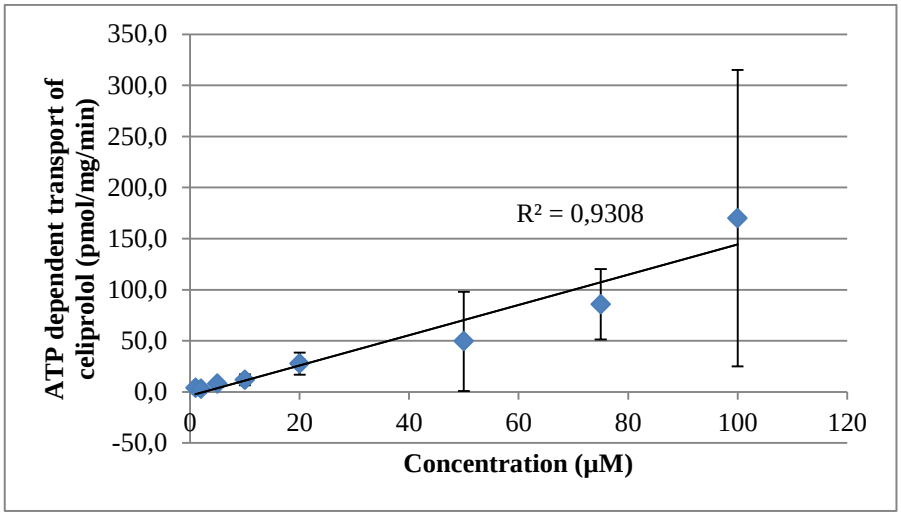


Figure 15. ATP dependent transport of celiprolol with concentrations of 1-75 µM and incubation time 1 min (Average ± SD)

The transport of aliskiren was determined with concentrations of 0.5-30 µM but aliskiren failed to show ATP-dependent transport when concentration increased over 2 µM. With aliskiren concentration of 4 µM passive transport was 1.3-fold compared to ATP- dependent transport. The transport rate decreased when concentration of aliskiren increased (Figure 16).

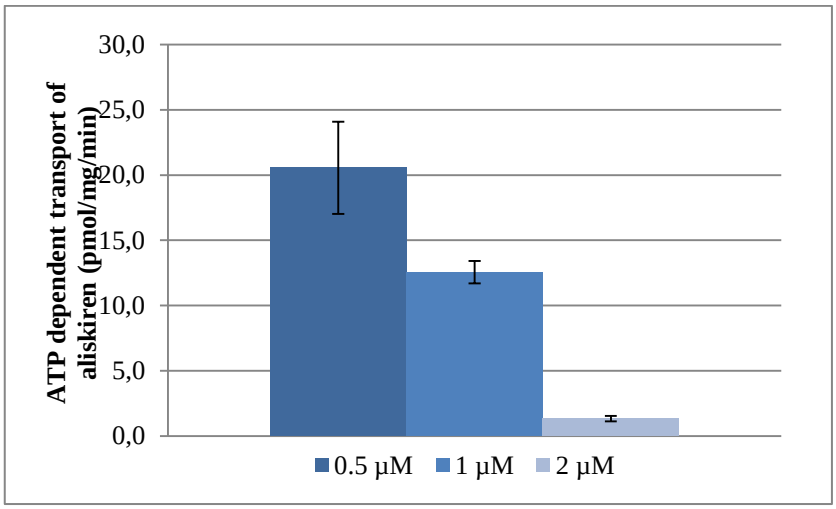


Figure 16. ATP dependent transport of aliskiren (nmol/mg/min) decreased when concentration of aliskiren increased. Incubation time was 1 minute (Average ±SD)

9.2 Cell assay

9.2.1 Permeability of control compounds and TEER

Results from paracetamol cell transport experiment for each well are presented in Appendix 3. The average apparent permeability of paracetamol from apical to the basolateral side and the average mass balance of the paracetamol wells are presented in Table 11.

Table 11. Transport of paracetamol 50 µM (average ± SD, n=2, passage 13) from the apical to the basolateral side (A-B) and mass balance. The samples were collected at 30, 60, 90 and 120 min after initiation of the experiment.

Substrate, concentration	**$P_{app\ x10}^{-6}$ (cm/s) A-B**	**Mass balance % ± SD A-B**
Paracetamol, 50 µM	17.2 ± 0.1	103 ± 0.14

Permeability test of Lucifer yellow was performed only from the apical to the basolateral side and concentration was determined in four time points (30-120 minutes). Transport of Lucifer yellow in single wells is presented in Appendix 5. The average permeability of Lucifer yellow was from 0.047 ± 0.025 to 1.2 ± 1.1 $x10^{-6}$ cm/s when the cell passage number varied from 12 to 18 (Table 12). The difference between the highest and lowest permeability value was about 25-fold. The permeability increased with increasing passage number. The apparent permeability values in the experiment with tests compounds varied from 0.047 ± 0.025 to 1.0 ± 0.28 $x10^{-6}$ cm/s.

Table 12. P_{app} values of Lucifer yellow (250µM) in MDCKII-MDR1-cells with different passage numbers. Test was performed from the apical to the basolateral side

Passage number	**P_{app} $x10^{-6}$ (cm/s)**
12	0.077
13	0.72, 0.057

14	0.047
15	0.48
16	0.13
17	1.0
18	1.2

The permeability of Lucifer yellow increased when concentration of DMSO increased (Figure 17). When DMSO concentration was 5 % the permeability of Lucifer yellow increased 2.7-fold compared to well where DMSO concentration was 0 %. Additionally DMSO concentration of 0.1 % and 0.2 % increased Lucifer permeability 2.1- and 2.3 fold, respectively.

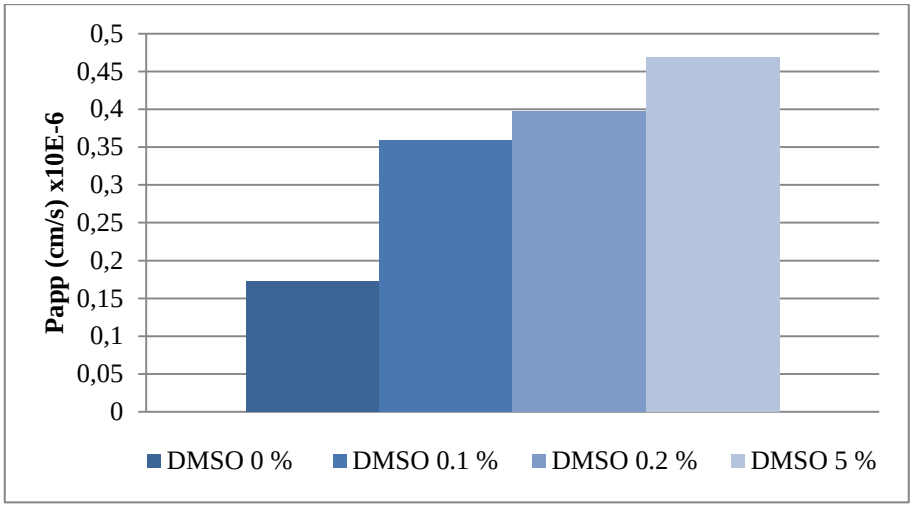


Figure 17. Effect of DMSO (dimethyl sulfoxide) concentration (0-5 %) on apparent permeability (P_{app}) of Lucifer yellow (250 µM) (n=1-2, passages 13 and 16).

The average (±SD) TEER value during P-gp substrate experiments in the buffer before the experiment was 62 ± 12 Ωcm^2 and after the experiments 61 ± 8 Ωcm^2. The TEER values did not have great variation between experiment and no significant drop of the values was detected after the experiment. TEER values during each experiment are presented in Appendix 6. TEER values were measured in growth media on day 4 and 5 after the seeding but no significant increase was detected on day 5 compared to day 4.

The average TEER values in the media on 4th day and 5th day were 77 ± 6 Ωcm^2 and 70 ± 9 Ωcm^2, respectively.

9.2.2 Permeability of substrates

The average apparent permeability values of P-gp substrates alone are presented in Table 13. Results from single wells are presented in Appendix 1. Celiprolol demonstrated the highest apparent permeability (P_{app}) while aliskiren demonstrated the lowest apparent permeability. Aliskiren failed to show results with concentration 2 µM and K_m determination due low recovery to the other side of the membrane. However permeability was obtained with 30 µM from the apical to the basolateral side. Talinolol showed the highest efflux ratio and celiprolol the lowest.

Table 13. Apparent permeability (P_{app}) of the test compounds through MDCKII-MDR1 cell monolayer from the apical to the basolateral (A-B) side and from the basolateral to the apical side (B-A), recovery of test molecules after experiment (average % ± SD) and efflux ratios. The samples were collected at 30, 60 and 90 min (fexofenadine, n= 3, passage 13), at 30, 60, 90 and 120 min (talinolol, celiprolol, n=3, passages 14 and 13, respectively) or at 60, 90 and 120 min (aliskiren, n=2, passage 17) after initiation of the experiment.

Substrate	**P_{app} x10^{-6} cm/s ± SD**		**Efflux ratio**	**Recovery % ± SD**	
	A-B	**B-A**		**A-B**	**B-A**
Fexofenadine 10 µM	0.19 ± 0.12	0.57 ± 0.13	3.0	94 ± 11	88 ± 66
Talinolol 10 µM	0.25 ± 0.08	3.6 ± 0.79	14	94 ± 9	101 ± 15
Celiprolol 10 µM	0.50 ± 0.30	0.85 ± 0.31	1.7	79 ± 3	70 ± 1
Aliskiren 30 µM	0.056 ± 0.002	-	-	191 ± 3	-

9.2.3 Permeability of substrates with inhibitors

The average apparent permeability values of the P-gp substrates with P-gp inhibitors itraconazole and cyclosporine are presented in Table 14. Results for single wells are presented in Appendix 2. Permeability of aliskiren (2 µM) with itraconazole was not obtained because of the low recovery of aliskiren on the receiver side of the membrane. Itraconazole had no effect on celiprolol or fexofenadine efflux ratio. Permeability of celiprolol to the absorptive direction is the same with and without itraconazole but permeability of fexofenadine is slightly changed with itraconazole. Celiprolol transport in the absorptive direction was clearly increased when administered with cyclosporine compared to the administration alone or with itraconazole. The A-B transport of celiprolol with cyclosporine was even higher than B-A transport of celiprolol alone. Also A-B permeability of talinolol was increased when administered with cyclosporine however remained clearly lower than B-A permeability of talinolol alone.

Table 14. Apparent permeability (P_{app}) of test components with inhibitors through MDCKII-MDR1 cell monolayer from the apical to the basolateral side (A-B) and from the basolateral to the apical side (B-A), recovery of test molecules after experiment (average % ± SD) and efflux ratios (ER). The samples were collected at 30, 60 and 90 min (fexofenadine and celiprolol, n= 2-3, passage 13) or at 30, 60, 90 and 120 min (talinolol, n=3, passage 14)

Substrate	**Inhibitor**	**P_{app} x10^{-6}cm/s ± SD**		**ER**	**Recovery % ± SD**	
		A-B	**B-A**		**A-B**	**B-A**
Fexofenadine 10 µM	Itraconazole 10 µM	0.26 ± 0.16	0.75 ± 0.44	2.9	93 ± 4	117 ± 13
Celiprolol 10 µM	Itraconazole 10 µM	0.53 ± 0.20	0.96 ± 0.29	1.8	93 ± 5	79 ± 5
Celiprolol 10 µM	Cyclosporine 10 µM	1.65 ± 1.74	0.46 ± 0.64	0.3	79 ± 3	67 ±3
Talinolol 10 µM	Cyclosporine 10 µM	0.76 ± 0.63	-	-	86 ± 13	-

10 DISCUSSION

10.1 Feasibility of vesicle transport assay

Celiprolol and aliskiren are more hydrophilic molecules than talinolol and fexofenadine which gives reason to assume they would be better subjects for vesicular transport assay. Hydrophobic molecules tend to absorb to membranes and therefore make it more difficult to predict the transport in membrane vesicles (Giacomini et al. 2010). Another problem is high to medium permeability subjects escaping from inside the vesicles (Glavinas et al. 2008). In agreement with this hydrophilic aliskiren demonstrated higher ATP dependent transport rates than fexofenadine and talinolol (1 µM, 1 min). In addition talinolol failed to show active transport with higher concentrations (10 and 100 µM) and therefore appeared to be poor subject for the vesicle transport assay. Fexofenadine showed the highest ratio between ATP dependent and passive transport and the highest transport rate with incubation time of 1 min which could mean that it escapes from vesicles when incubated for longer period. On the other hand celiprolol showed low efflux transport rate even though it is the most hydrophilic drug. This however is probably because of the low P-gp affinity but it also could also support the theory that P-gp substrates are hydrophobic and molecules enter to the P-gp binding site through hydrophobic gate.

Celiprolol showed non– saturable kinetics up to 100 µM from which can be concluded that K_m-value of celiprolol is over 100 µM. This finding is rational because Lipka et al. (1998) determined 10-fold higher K_m-value of 1±0.23 mM for celiprolol in caco-2 cells. Aliskiren failed to show ATP dependent transport with concentrations over 3 µM. This is in disagreement with another master's thesis work where K_m-value of aliskiren was successfully determined (Koskenkorva 2012). The highest transport rate accomplished for aliskiren was 14 pmol/mg/min (2 µM) which is significantly lower than V_{max} value of 890 pmol/mg/min determined in the previous work.

In this thesis cholesterol was added to the Sf9 membrane vesicles containing cholesterol in order to enhance function of P-gp which could have affected to the results. The

cholesterol content of Sf9-cells is lower than in mammal cells and cell cholesterol content cholesterol have been suspected to have an effect on activity of ABC transporters (BCRP, P-gp) (Kalsson et al. 2010, Pál et al. 2007). There is also evidence that cholesterol can affect the ATPase activity of P-gp (Eckford and Sharom 2008, Garrigues et al. 2002). Garrigues et al. (2002) demonstrated slightly higher P-gp ATPase activity in the presence of 20 % cholesterol compared to 0 and 30 % in DMPC proteoliposomes.

Partitioning of substrates in to the lipid layer (egg PC vesicles) has been demonstrated to be dependent on cholesterol concentration (Eckford and Sharom 2008). Distribution of vinblastine between membrane and water (the membrane-water distribution coefficient, K_{lip}) was highest without cholesterol and decreased about 6-fold when cholesterol concentration increased to 20 % (w/w). Also the K_{lip} of verapamil, daunorubicin, Hoechst 33342 and rhodamine 123 decreased with increasing cholesterol concentration. In addition the affinity of the substrate to P-gp was decreased with increasing cholesterol concentration. This could explain why the K_m- value of aliskiren was not managed to obtain in this thesis although Koskenkorva (2012) determined K_m value of 5.05 µM for aliskiren with Sf9 vesicles without added cholesterol. Aliskiren showed very low permeability (0.056 x10-6 cm/s) in the MDCKII-MDR1 cells and the increased rigidity of the lipid layer could interfere with the distribution of aliskiren to the membrane and access inside the vesicles. As presented in the literature review the substrate binding happens in the lipid bilayer or from the cytosol. The fact that aliskiren transport is affected by cholesterol could mean that binding of aliskiren occurs in the lipid bilayer.

As can be seen from Table 9 and Table 10 the detected concentrations inside the vesicles were low (magnitude of nM) even though powerful detection technique was used. This is one of the reasons why results obtained from vesicle assay are variable. In this work the ratio between passive and active transport was only 1.6–2.6 which is why it is difficult to separate ATP-dependent and passive transport and see the effect of an inhibitor. Therefore according to the current study using vesicle assay for P-gp interaction studies is not possible because detecting small effect of inhibitor to substrate

transport rate would be very challenging. Vesicle assay could be used to recognize P-gp substrates. However the deviations of the vesicle assay results are great thus it cannot be said certainly which compounds are P-gp substrates.

10.2 Quality tests for permeability assays

In the current study the fluctuation of Lucifer yellow permeability values was great. Upper limit for Lucifer yellow P_{app} employed in this thesis was 1.0 $x10^{-6}$ cm/s. The passages 13, 14, 16 and 17 used in the experiments P_{app} values varied from 0.047 to 1.0 $x10^{-6}$ cm/s. In one previous study with MDCKII-MDR1 cells of Lucifer yellow permeability limit values were 0.1 to 0.7 $x10^{-6}$ cm/s (Zhang et al. 2006). The fluctuation in the current study was much greater and the maximum value higher than in the study of Zhang et al. The difference between the lowest and highest Lucifer yellow P_{app} value was approximately 20-fold which could have affected the quality of the results. Probably the variation of cell monolayer permeability has the most considerable effect on celiprolol permeability because it is the most hydrophilic test drug. The formation of the MDCKII-MDR1 cell monolayer has been suggested to have failed if Lucifer yellow permeability is over 0.5 $x10^{-6}$ cm/s (Cyprotex 2013). This limit was exceeded in celiprolol and aliskiren experiments. In theory it is therefore possible that permeability of the two molecules is higher because of the leakier cell layer. All in all Lucifer yellow P_{app} varied from 0.047 to 1.2 $x10^{-6}$ cm/s with passages 12-18. Experiment results where Lucifer yellow P_{app} was over $1x10^{-6}$ cm/s were discarded. The rapid increase in Lucifer yellow P_{app} values could be result from mycoplasma contamination which is difficult to observe visually. Mycoplasma bacteria contamination can affect the cell growth and therefore affect forming of the uniform cell monolayer (Ryan 1994).

Concentration of DMSO seemed to have a moderate effect on permeability of Lucifer yellow. DMSO is a polar organic solvent which can penetrate biological membranes and therefore can increase the permeability of the cell monolayer. As expected the highest permeability resulted from DMSO concentration of 5 % and gave about 3-fold increase compared to 0 % DMSO. The maximal concentration used in the experiment was 0.6 %. The permeability difference was 2.3-fold between 0 % and 0.2 % which was

the DMSO concentration in most of the experiment. Maximal DMSO concentration 0.6 % was used in aliskiren K_m experiment. Furthermore the difference in Lucifer yellow controls between experiments was even 20-fold which implicates that effect of DMSO is minor compared to the effect of increasing passage number. According to Absorption Systems the test solution DMSO concentration should not exceed 0.8 % (Absorption systems 2013). This is in agreement with the current study.

TEER values were rather low during the experiments. The average TEER value in the buffer before, and after the experiment was 62 ± 12 Ωcm^2 and 61 ± 8 Ωcm^2, respectively. In one previous study the average TEER value of the MDCK-MDR1 cell monolayer cultured on polycarbonate was 126 Ωcm^2 (Williams et al. 2003). The values in the current study were lower which could indicate that there have been some problems with cell growth. Zhang et al. (2006) measured TEER values of 120-160 Ωcm^2 for MDCKII-MDR1-cells that had been grown on polycarbonate membrane. The origin of the cells in the experiment of Zhang et al. and current study was the same (Netherlands Cancer Institute) but the passage was higher in the current study (12-19 compared to 5-9) which could explain the difference. No significant increase was detected between the values measured one day before (4^{th} day after the seeding) the experiment compared to the values measured just before the experiment (5^{th}) day after the seeding) which is why it was decided to perform tests on 5^{th} day after seeding the cells on membranes. TEER did not show clear downward trend with increasing passage number although Lucifer yellow shower clear difference. From this can be concluded that Lucifer yellow is more reliable indicator of cell integrity but TEER value measurement is an easy method for identifying broken cell layers during experiments.

P_{app} of paracetamol determined in this study is in agreement with previous studies. Permeability coefficient determined for paracetamol was 17.9 $x10^{-6}$ which is very close to a previous determined value 17.4 $x10^{-6}$ cm/s (A-B) (Cyprotex 2013). According to the BCS classification a drug is classified as a low permeable drug according its permeability and bioavailability (WHO 2005, FDA 2009). High permeable drug should have 90 % absorption from the intestine. According to previous studies the bioavailability of studied P-gp substrates is very low (Table 5). Reference drug

paracetamol is on the edge of high and low permeable drug and it has been classified as BCS I or III drug (WHO 2005, FDA 2009). The P_{app} values (A-B) of all the test drugs were all below the P_{app} of paracetamol thus based on these *in vitro* tests aliskiren, talinolol, celiprolol and fexofenadine can be classified as low permeability drugs.

The variation between parallel samples was great in part of the experiments which needs to be considered in discussion. In addition number of parallel samples was very low (1-3) which is why calculating average and standard deviation does not necessarily show actual deviation. Part of the samples were diluted for the analysis which causes some inaccuracy for the analysis results which could be one of the reasons why recovery of compounds after cell experiment was low or in some cases irrationally high. The analysis method for P-gp substrates was not validated (accuracy and repeatability were not tested) because of the lack of resources thus it is not possible to evaluate reliability of analysis results. Also one problem was that samples were analyzed after variable time and compound could have decomposed during the storage.

10.3 *In vitro* P-gp mediated efflux of test drugs

The results from this thesis suggest that all the test drugs are transported with active mechanism. Fexofenadine, talinolol and celiprolol showed efflux ratio over 1 in MDCKII-MDR1 cells. According to FDA (2012) a drug is probably a P-gp substrate if its net efflux ratio is equal to 2 or higher. In this thesis talinolol and fexofenadine had an efflux ratio higher than 2 thus they fulfilled the FDA criteria. The efflux ratio of celiprolol was only 1.7 while no efflux ratio was managed to determine for aliskiren. On the other hand all the test drugs showed at least weak ATP dependent transport in the membrane vesicles. The amount of other transporter proteins beside P-gp in the used vesicles is low thus the active transport is probably mediated by P-gp (Sarkadi et al. 1992).

Further if also the efflux ratio of the test drug is decreased to half or to unity in the presence of known P-gp inhibitor, it is proven to be P-gp substrate (FDA 2012). In this

thesis the efflux ratio of celiprolol and fexofenadine was determined with P-gp inhibitor itraconazole. According to the result neither of the drugs seemed to be P-gp substrates because their efflux ratios were unaltered. Only fexofenadine permeability to the absorptive direction was very slightly increased (**Error! Reference source not found.**) hich resulted to 0.1 unit decrease in efflux ratio. However it is surprising that transport to the secretive direction is increased with both celiprolol and fexofenadine in the presence of itraconazole. More repetitions are needed to see if this is an analysis error or caused by inhibition of active transport.

Saturation of the transport could explain the lack of inhibitor effect on permeability of celiprolol and fexofenadine. However used test concentrations (10 µ) were well below the K_m-values of fexofenadine (150 µM) and celiprolol (1 mM) thus saturation of transport of these two drugs can be excluded (Karlsson et al. 1993, Petri et al. 2004). Another possible explanation for the absence of inhibitory effect is that the inhibitor is not present at concentration high enough to inhibit the binding of substrate to P-gp. Itraconazole concentration of 10 µM was used in all the experiments. In one previous work itraconazole concentration of 2.8 µM inhibited transport of cimetidine in MDCKII-MDR1 cells (Karyekar et al. 2003). Itraconazole inhibited also transport of digoxin with IC50 value of 1.3 µM in MDCKII-MDR1 cells (Keogh and Kunta 2006). On these grounds can be assumed that the concentration of 10 µM is sufficient. Itraconazole is metabolized by liver enzymes thus it is possible that *in vivo* inhibition is mediated by its metabolites (drugs@FDA). The metabolism is probably not occurring in MDCKII-MDR1 cells because there are very few metabolizing enzymes expressed in MDCKII-MDR1 cells (Quan et al. 2012). However since itraconazole has been shown to inhibit P-gp in MDCKII-MDR1 cell previously this explanation is not likely.

In one previous study (Karyekar 2003) the cells were pre-incubated for 30 min with inhibitor while in this study the inhibitor and substrate were added at the same time. By pre-incubation with inhibitor can be ensured that inhibitor is present at P-gp binding places. It is possible that diffusion of the larger inhibitor (itraconazole molecular mass 705.6 g/mol) can be slower in the cell membrane and therefore requires more time to get to the binding place. Itraconazole has also high lipophilicity (predicted $logD_{7.4}$ 4.29)

and it is insoluble in the water (Micromedex 2.0, ACD labs 8.08). Itraconazole stock concentration was prepared in DMSO without any problems and further diluted to aqueous transport buffer. However, the solubility was not controlled instrumentally thus it is possible that itraconazole was not soluble in test solution which would have prevented inhibitory the function.

10.3.1 Transport of fexofenadine

Fexofenadine (10 µM) P_{app} value 0.19 x 10^{-6} cm/s in the absorptive direction (A-B) was close to a fexofenadine (50 µM) P_{app} value of 0.17 x 10^{-6} cm/s determined earlier in Caco-2 cells (Petri et al 2004). On the other hand, the value for secretive direction (B-A), 0.57 x 10^{-6} cm/s, differed greatly from the value of 14.22 x 10^{-6} cm/s Petri and coworkers determined. Therefore also efflux ratio determined in this work was also significantly lower (3.0 compared to 85). In Caco-2 cells, which Petri et al. used in their experiments, also other transporters are expressed in addition to P-gp. Fexofenadine has been suggested to be transported also by MPR2 and OATP transporters (Sasaki et al. 2004, Sakugava et al. 2009, Akamine et al. 2012). The cellular uptake of fexofenadine was mediated by human OATP (K_m 6.4 µM), rat Oatp1 (32 µM) and Oatp2 (6 µM**)** transporters in HeLa cells (Cvetkovic et al. 1999). The MPR mediated efflux along with P-gp mediated efflux in the caco-2 cells could explain the high transport rate to B-A direction. In addition OATP-mediated influx would explain why A-B transport is equal in current study and study of Petri et al. (2004) (despite the higher efflux caused by two efflux proteins).

10.3.2 Transport of celiprolol

In the study of Karlsson et al. (1993) transport of celiprolol (50 µM) in the secretive direction was 5 times higher than transport in the absorptive direction in Caco-2 cells. Efflux ratio of celiprolol in this work was only 1.7 which indicates low P-gp mediated transport. However there was great variation in permeability values of celiprolol when studied alone and with cyclosporine and recovery in those experiments was low which

causes uncertainty in the results. When celiprolol was studied with cyclosporine the efflux ratio determined here decreased under unity (to 0.3 from 1.7) (Table 14) which could be result from an active uptake. Several studies suggest that celiprolol is OATP substrate Lilja et al. 2003, Lilja et al. 2004a, Lilja et al. 2004b, Uesawa and Mohri 2008, Kato et al. 2009. Significantly lower K_m value was determined for celiprolol OATP-A mediated uptake in Xenopus Laevis oocyte than K_m value for celiprolol secretion determined in caco-2 cells which refers to a greater affinity of celiprolol for the OATP than P-gp (Karlsson et al. 1993, Kato et al. 2009). However cyclosporine is also an inhibitor of OATPs, BCRP and MPR transporters thus it probably inhibited all these transporters (Agarwal et al. 2007, FDA 2012, Hillgren et al. 2013).

10.3.3 Transport of talinolol

Efflux ratio obtained from MDCKII-MDR1 cell experiment show here clearly that talinolol is a P-gp substrate. In this study the talinolol (10 µM) P_{app} (A-B) was 0.25 $x10^{-6}$ cm/s and P_{app} (B-A) 3.60 $x10^{-6}$ cm/s. Deferme et al. (2002) demonstrated P_{app} values of 1.08 $x10^{-6}$ cm/s and 11.74 $x10^{-6}$ cm/s for talinolol (100 µM) in Caco-2 cells for absorptive and secretive directions, respectively. The permeability coefficients of talinolol were clearly lower in this thesis but the efflux ratio was of the same magnitude (14 compared to 11). Also the P_{app} (B-A) increased about 3-fold by addition of P-gp inhibitor as in the study of Deferme et al. Lower expression of P-gp could be the reason why P_{app} values in this study are lower than in the study of Deferme's group. Also lower test concentration explains the difference and it also seems that talinolol could have higher K_m than 100 µM because efflux ratio is the same as with concentration 10 µM implicating that transport has not saturated.

10.3.4 Transport of aliskiren

Aliskiren concentration (2 µM) used in the cell experiment was too low thus transported amounts were undetectable. P_{app} value (A-B) was managed to determine with concentration 30 µM. K_m value determined in membrane vesicles extracted from P-gp

expressing Sf9 cells was 5.05 µM (Koskenkorva 2012). Therefore it is possible that P-gp mediated transport is saturated and P_{app} is the passive permeation. The results show that aliskiren has very low absorption (0.056 $x10^{-6}$ cm/s) which implicates that it is more sensitive for the effect of transporters because the transporter has time to remove the drug from the cell membrane before new drugs penetrates through (Giacomini et al. 2010). This explains why in vivo effect of P-gp inhibitor on aliskiren pharmacokinetics is great.

10.4 Correlation of *in vivo* effect with *in vitro* results

Correlation between *in vitro* and *in vivo* effect of P-gp inhibition can not be calculated because test compounds were not tested with an inhibitor in the vesicle assay test, and in the cell experiments permeability of test compounds (celiprolol and fexofenadine) was not affected by itraconazole. However there is some correlation between efflux ratios of substrates alone determined *in vitro* and increase of AUC *in vivo* when magnitude of the effect is compared between different substrates. Also some compounds had more clear ATP-dependent transport than other.

In the vesicle assay aliskiren showed the highest ATP dependent transport (14 pmol/mg/min) compared to other test drugs which is in agreement with its *in vivo* pharmacokinetics. Aliskiren AUC increased 6-fold with itraconazole while AUC of celiprolol and fexofenadine increases only 2-fold (Table 4). From aliskiren cell experiment results any conclusions cannot be made because the efflux ratio was not successfully determined. It would be interesting to repeat aliskiren experiments with higher concentration (2 µM was not detectable) to see if efflux ratio is higher than with celiprolol and fexofenadine.

Talinolol showed the highest ATP dependent transport with the vesicles (4.7 pmol/mg/min) after aliskiren and clearly greater transport to the secretive direction (efflux ratio 14). Also in vesicles the difference between passive and ATP-dependent transport was clear. *In vivo* results suggest that P-gp inducers increase the P-gp mediated transport of talinolol (Table 4). The *in vivo* effect of inducer is however low

(AUC decreases less than 50 %) compared to what could be expected according to efflux ratio determined in the current study when compared to efflux ratios determined to other substrates. For instance fexofenadine AUC increases 2-3-fold *in vivo* while in the current study it has efflux ratio 3.

When *in vivo* and *in vitro* cell experiment result of celiprolol and fexofenadine are examined it can be seen that they correlate with *in vivo* studies. According to cell experiments celiprolol (efflux ratio 1.8) absorption is less dependent on P-gp efflux than fexofenadine (efflux ratio 3). *In vivo* the effect of itraconazole is also minor to the AUC of celiprolol (AUC increases 1.8-fold). It must be noted however that drug recovery in celiprolol cell assay was low (B-A 70 % and A-B 79 %) which could have affected the results. Either way also in vesicle assay celiprolol showed lower ATP dependent transport rate compared to fexofenadine and talinolol, and low affinity to P-gp (K_m higher than 100 µM).

11 CONCLUSIONS

P-glycoprotein is expressed in organs which participate on drug absorption, distribution and elimination and P-glycoprotein mediates clinically significant interactions *in vivo*. The object of this thesis was to understand P-gp mediated drug interactions. In the literature part structure, function, regulation and substrate selection of P-gp was reviewed in order to understand complexity of P-gp. The structure of P-gp is studied widely and recent crystallization of mouse P-gp is big step towards knowing the structure of human P-gp. With the help of protein crystal structure P-gp interacting molecules can be screened computationally. Screening P-gp substrates and inhibitors is challenging because P-gp has several binding sites. When studied P-gp interactions with the help of computational methods the interactions should be screened by fitting each molecule combinations to the binding cavity.

In the experimental work P-gp mediated interactions of four P-gp substrates were studied with two *in vitro* methods and results were compared to *in vivo* interactions.

Vesicle assay method was able to show ATP dependent transport for all studied P-gp substrates in P-gp expressing vesicles. In addition in MDCKII-MDRII cells celiprolol, talinolol and fexofenadine showed an efflux ratio higher than 1 in which means that they have active transport. However there was no interaction detected between P-gp inhibitor itraconazole and P-gp substrates fexofenadine and celiprolol thus they do not meet the criteria of FDA for P-gp substrates. The reason for lack of interaction could be unsuccessful performance of the experiments (such as lack of pre-incubation with inhibitor) and not the lack of P-gp efflux.

According to results obtained here *in vivo* significance of drug interaction cannot be predicted. However some results obtained with *in vitro* vesicle assay, cell assay and *in vivo* studies are in line with each. Both vesicle assay and *in vivo* studies suggest that aliskiren is a good P-gp substrate while all the methods suggest that celiprolol is a poor substrate. I*n vitro* cell assay showed that talinolol was a good substrate for P-gp although *in vivo* results with inhibitors are not available. Fexofenadine seemed to be P-gp substrate in both *in vitro* assays in accordance with *in vivo* studies. According to this thesis vesicle assay and cell assay can be used to recognize P-gp substrates. However significance of P-gp mediated interactions is difficult to predict because of high variable results of both assays.

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APPENDIX 1

PERMEABILITY OF P-GLYCOPROTEIN SUBSTRATES IN MDCKII-MDR1 CELLS

Figure 1. Transport of fexofenadine 10 µM through MDCKII-MDR1 cell monolayer from the apical to the basolateral side (A-B) and from the basolateral to the apical side (B-A)

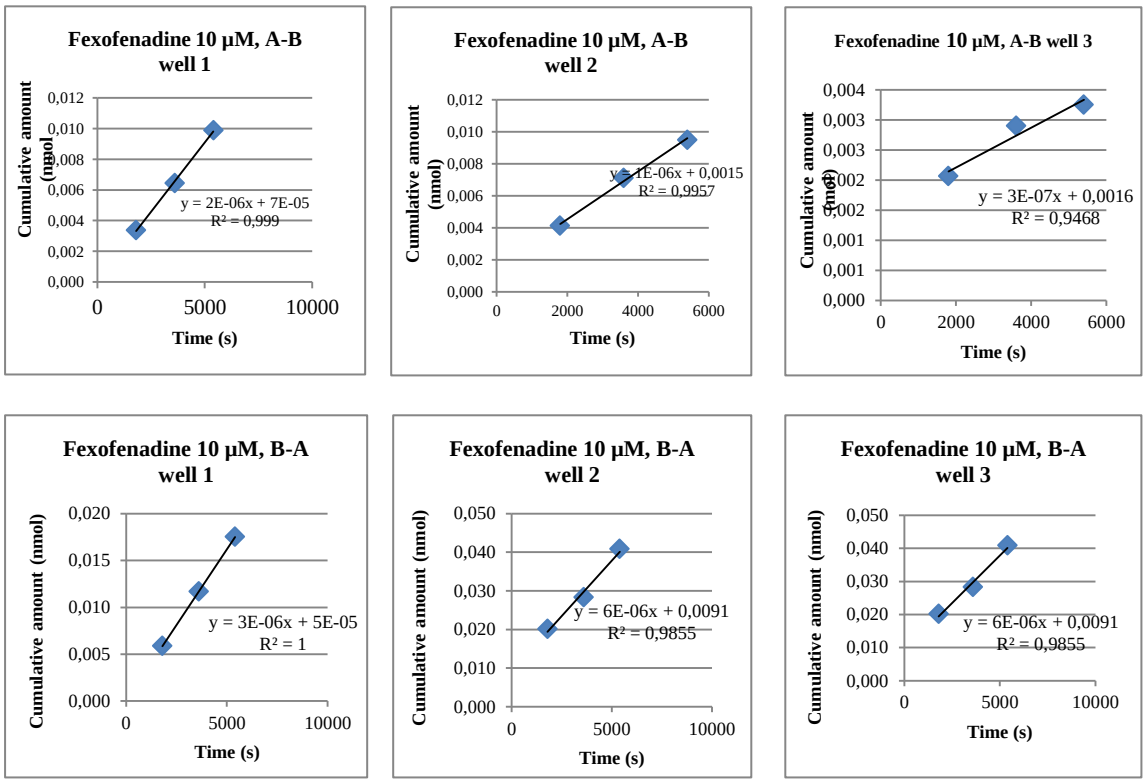

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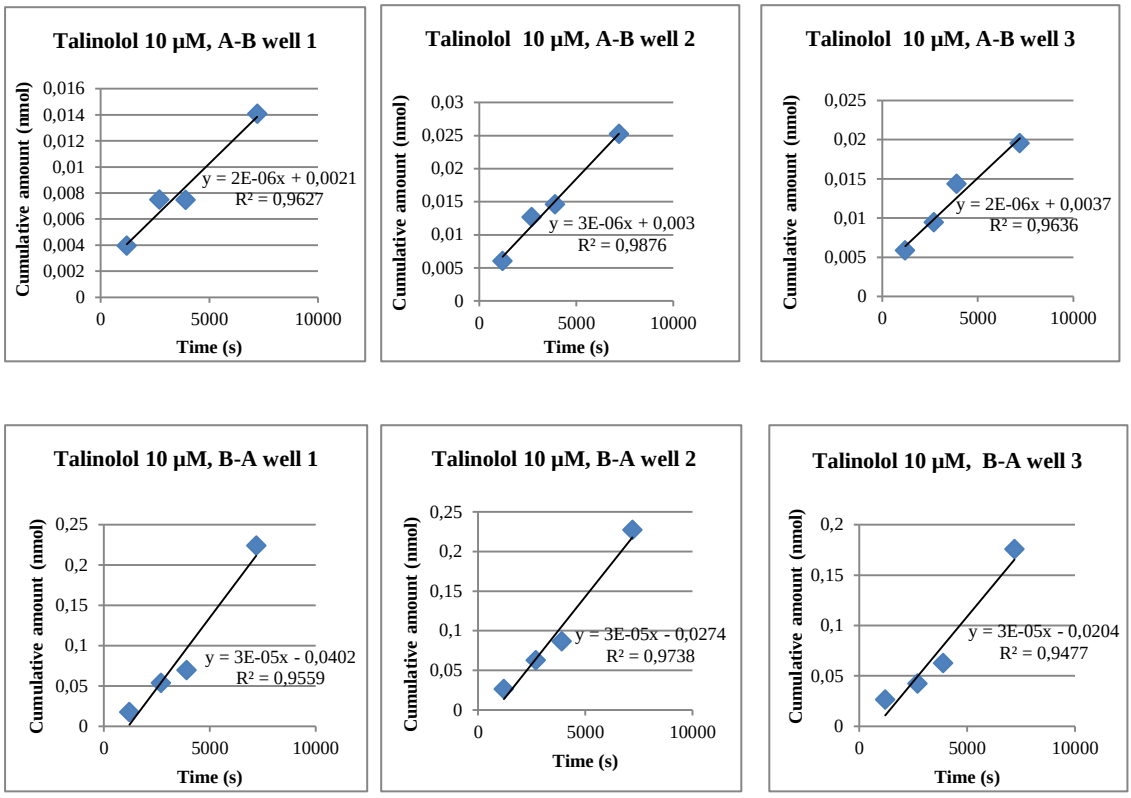
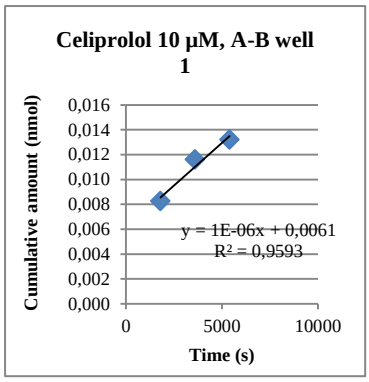
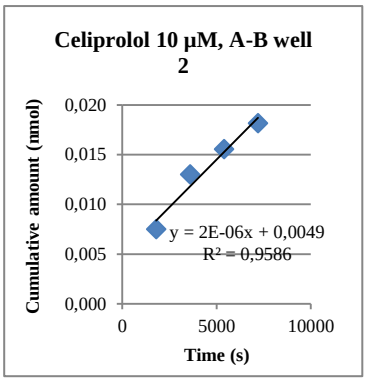
Figure 2. Transport of talinolol 10 µM through MDCKII-MDR1 cell monolayer from the apical to the basolateral side (A-B) and from the basolateral to the apical side (B-A)

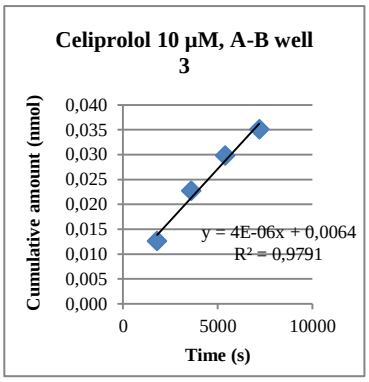


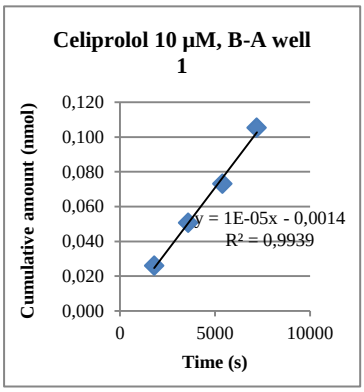
APPENDIX 1

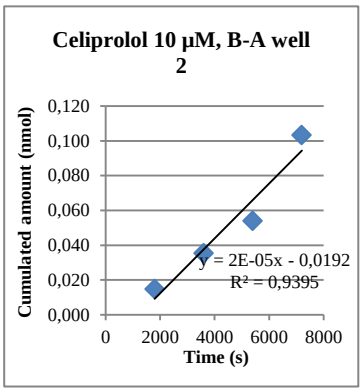
Figure 3. Transport of celiprolol 10 µM through MDCKII-MDR1 cell monolayer from the apical to the basolateral side (A-B) and from the basolateral to the apical side (B-A)











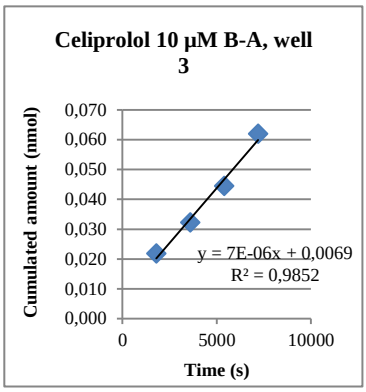


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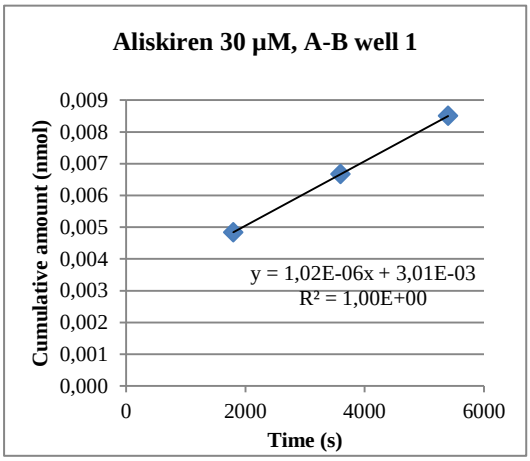
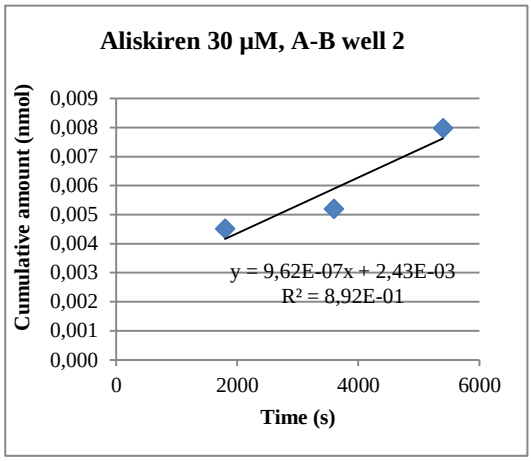
Figure 4. Transport of aliskiren 30 µM through MDCKII-MDR1 cell monolayer from the apical to the basolateral side (A-B)

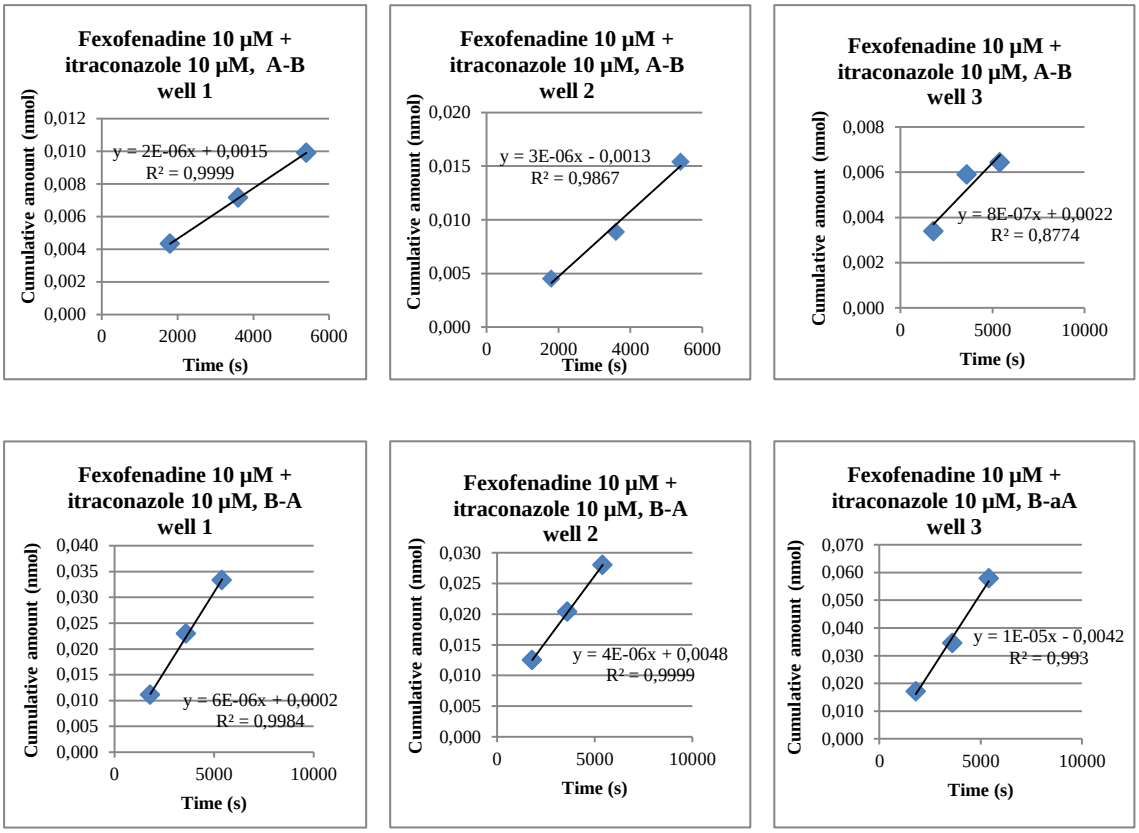




APPENDIX 2

PERMEABILITY OF P-GLYCOPROTEIN SUBSTRATES WITH INHIBITORS IN MDCKII-MDR1 CELLS

Figure 1. Transport of fexofenadine 10 µM with itraconazole 10 µM through MDCKII-MDR1 cell monolayer from the apical to the basolateral side (A-B) and from the basolateral to the apical side (B-A).



APPENDIX 2

Figure 2. Transport of celiprolol 10 µM with itraconazole 10 µM through MDCKII-MDR1 cell monolayer from the apical to the basolateral side (A-B) and from the basolateral to the apical side (B-A**)**

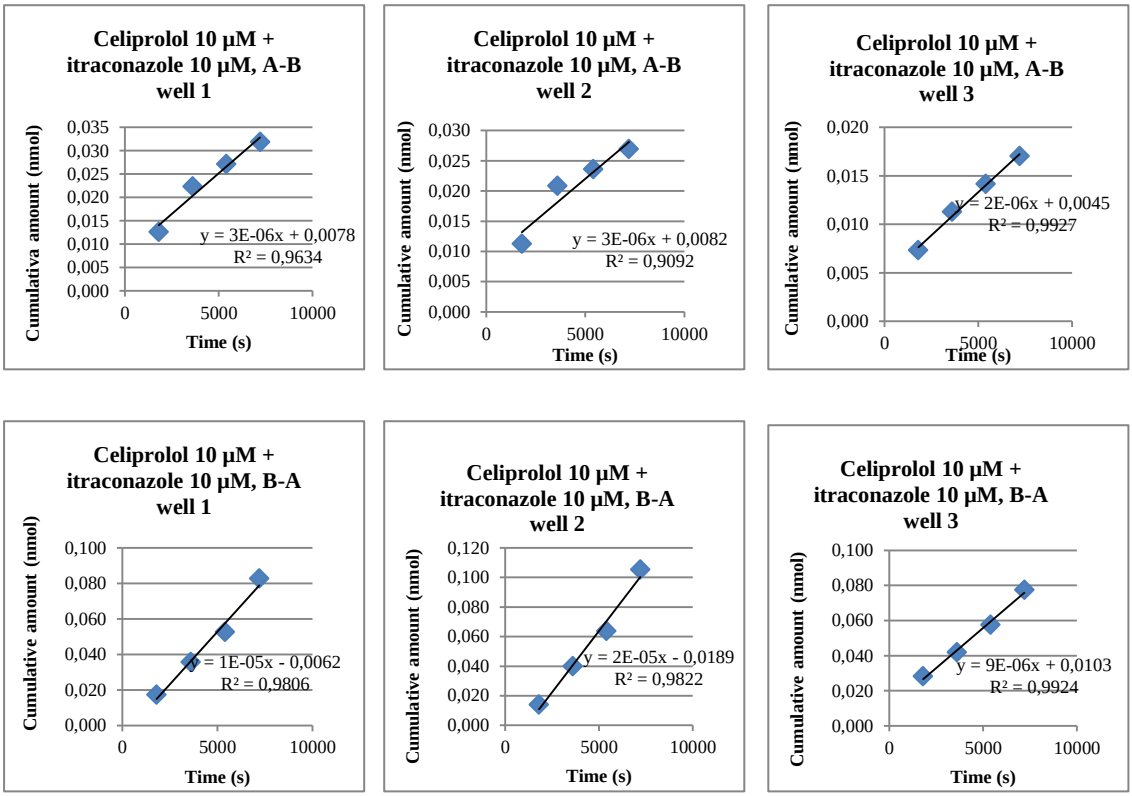


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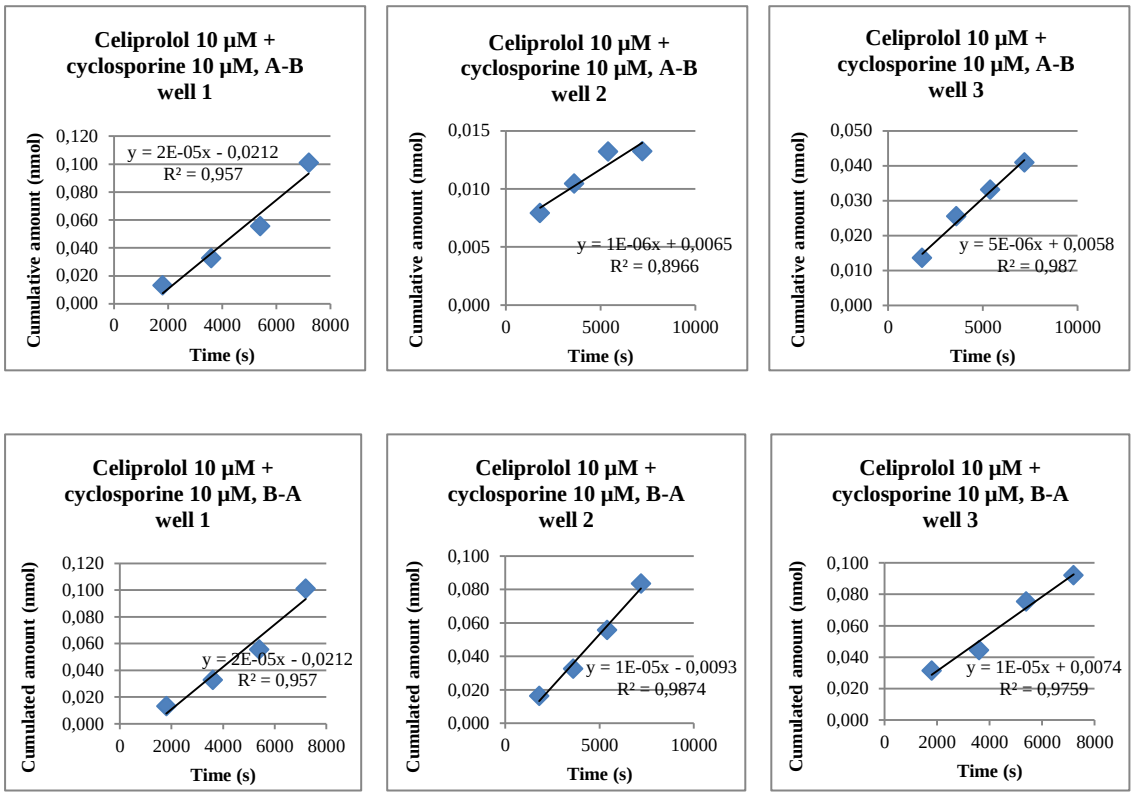
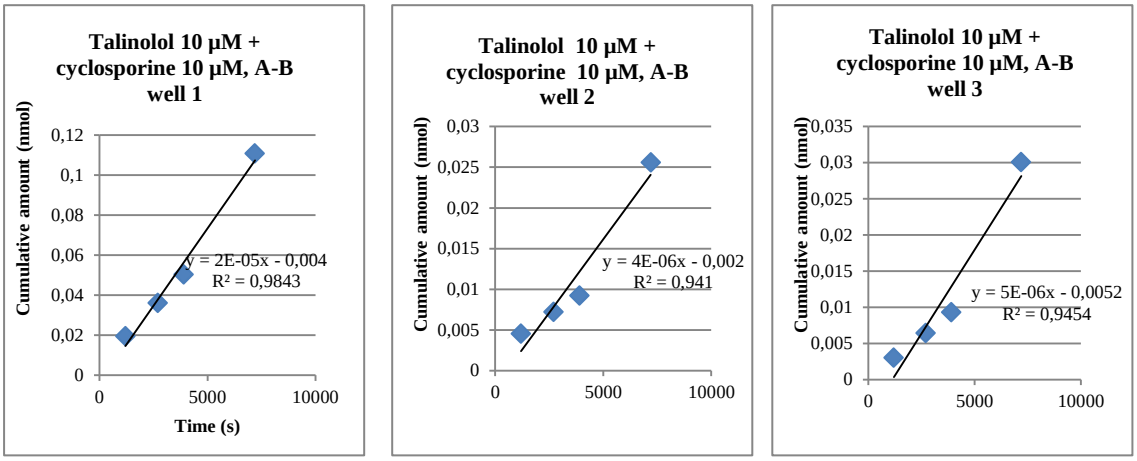
Figure 3. Transport of celiprolol 10 µM with cyclosporine 10 µM through MDCKII-MDR1 cell monolayer from the apical to the basolateral side (A-B) and from the basolateral to the apical side (B-A).



APPENDIX 2

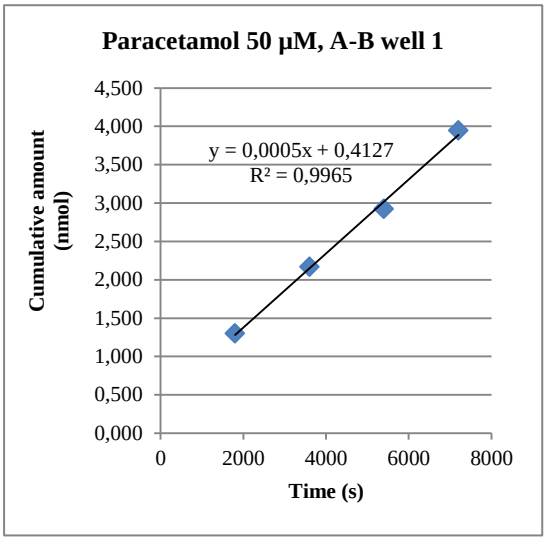
Figure 4. Transport of talinolol 10 µM with cyclosporine 10 µM through MDCKII-MDR1 cell monolayer from the apical to the basolateral side (A-B).

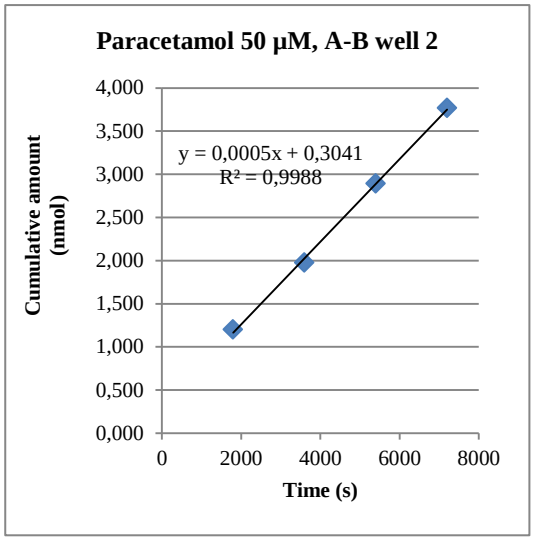


APPENDIX 3

PERMEABILITY OF PARACETAMOL IN MDCKII-MDR1 CELLS

Figure 1. Transport of paracetamol 50 µM from the apical to the basolateral side (A-B)

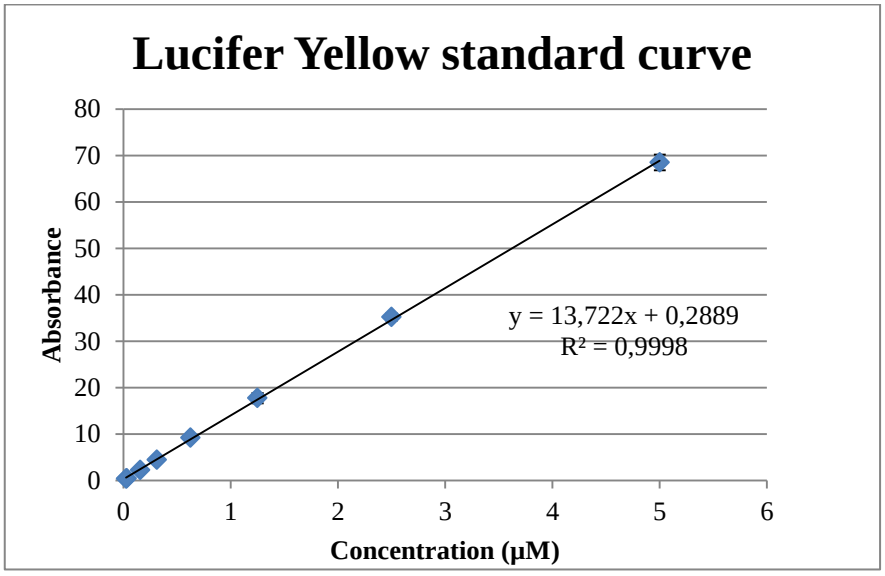




APPENDIX 4

LUCIFER YELLOW STANDARD CURVE

Figure1. Standard curve of Lucifer Yellow



APPENDIX 5

PERMEABILITY OF LUCIFER YELLOW IN MDCKII-MDR1 CELLS

Figure 1. Permeability of Lucifer yellow 250 µM from the apical to the basolateral side (A-B) during talinolol permeability determination.

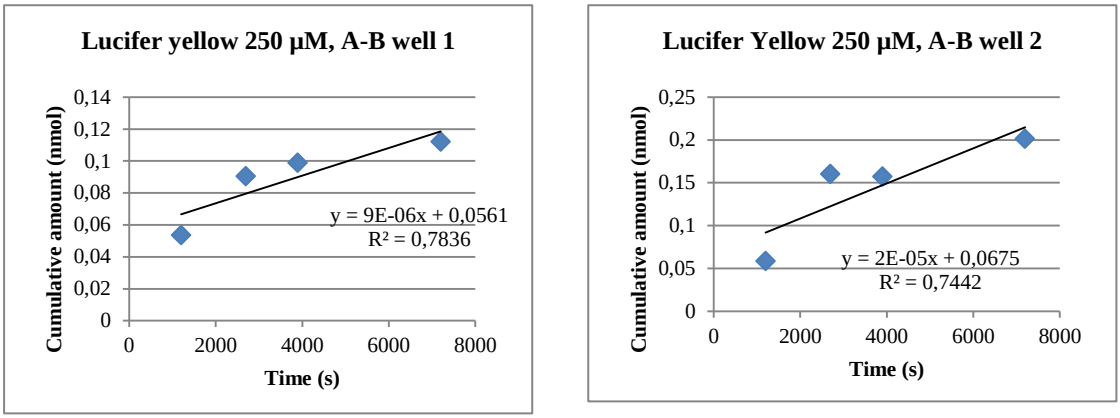
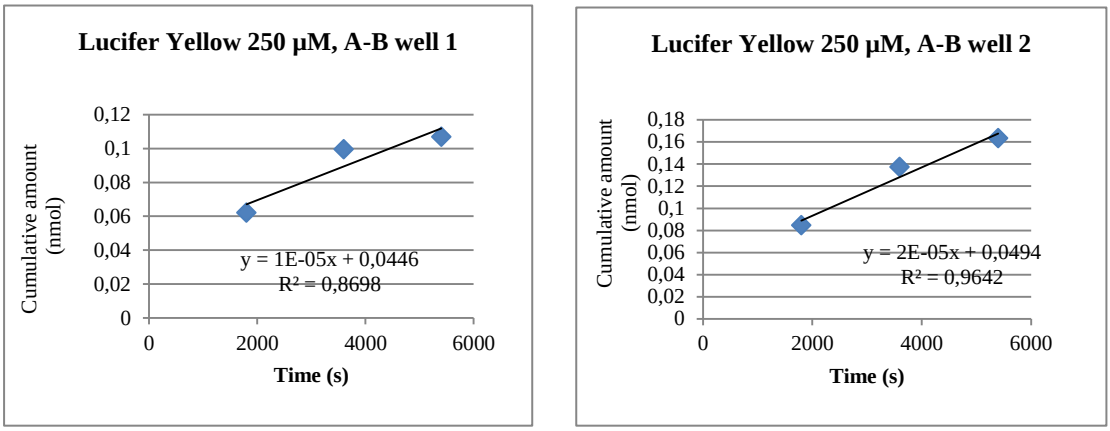


Figure 2. Permeability of Lucifer yellow 250 µM from the apical to the basolateral side (A-B) during fexofenadine and paracetamol permeability determination.



APPENDIX 5

Figure 3. Permeability of Lucifer yellow 250 µM from the apical to the basolateral side (A-B) during celiprolol permeability determination.

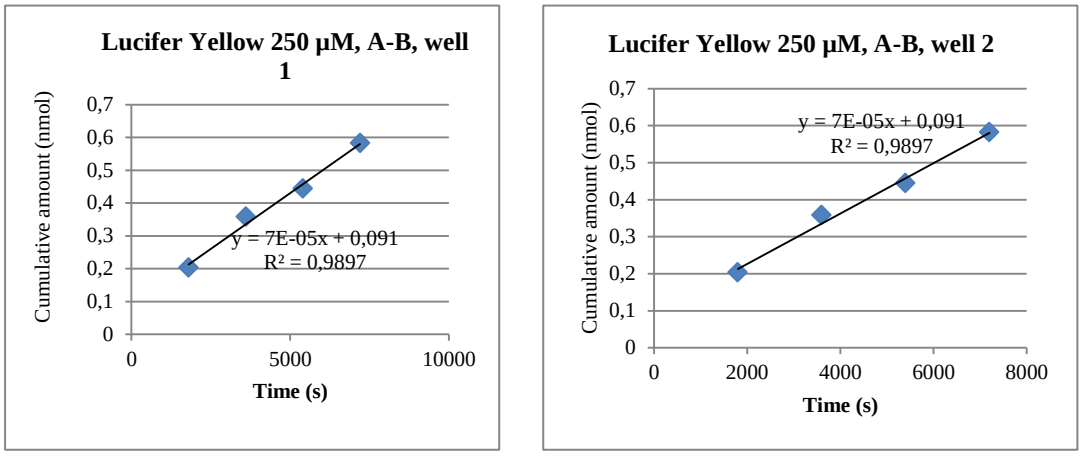


Figure 4. Permeability of Lucifer yellow 250 µM from the apical to the basolateral side (A-B) during aliskiren permeability determination

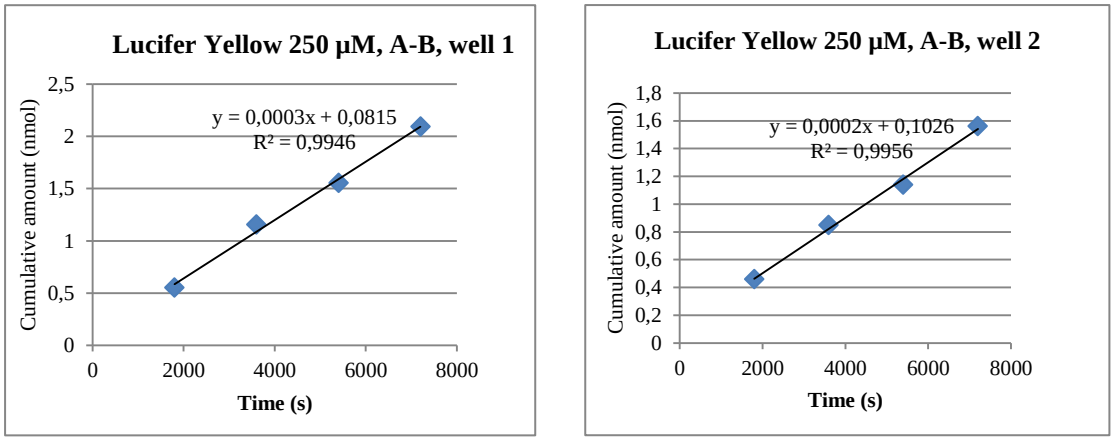


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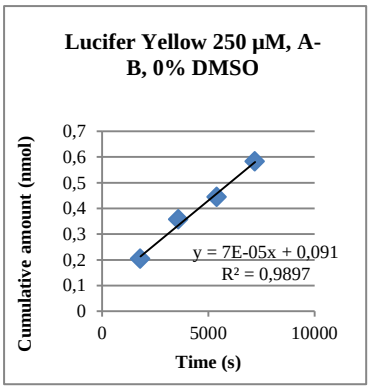
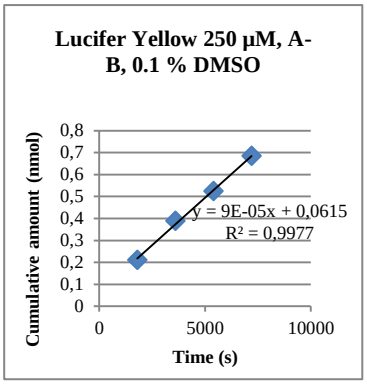
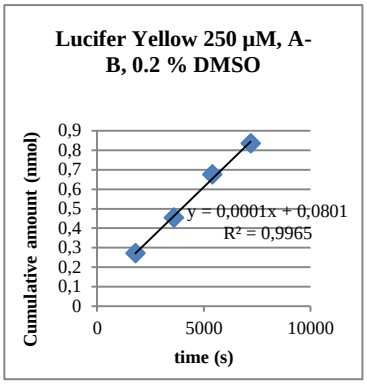
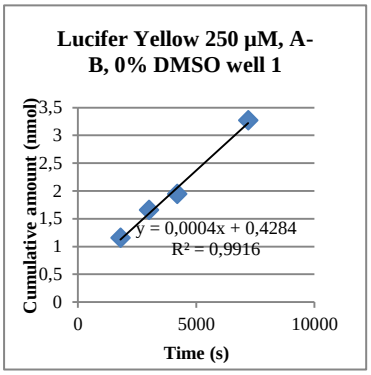
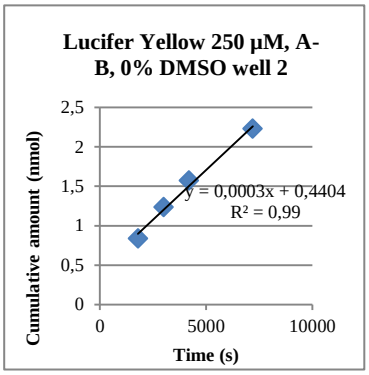
Figure 5. Permeability of Lucifer yellow 250 µM from the apical to the basolateral side (A-B) with DMSO concentrations (v/v) of 0%, 0.1%, 0.2% and 5%. (Upper row passage 13, lower rows passage 16)

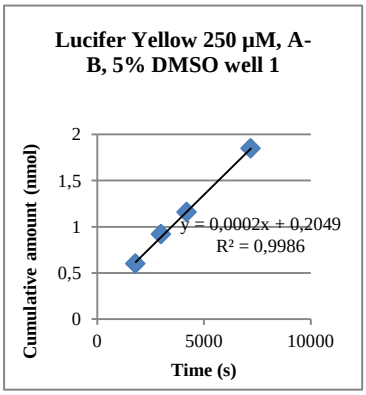


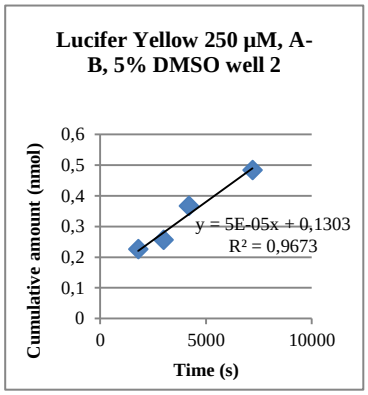












APPENDIX 6

AVERAGE TEER VALUES DURING CELL EXPERIMENTS

Figure 1. Average (±SD) TEER (trans-epithelial electrical resistance) values before cell experiments in cell culture media and transport buffer, and after experiments in transport buffer

Experiment	Before experiments TEER (Ωcm^2) (average ± SD)		TEER after experiment TEER (Ωcm^2) (average ± SD)
	Culture media	Transport buffer	Transport buffer
Talinolol	73 ± 16	-	75 ± 14
Celiprolol	87 ± 6	58 ± 7	71 ± 7
Aliskiren	53 ± 9	70 ± 6	59 ± 9
Fexofenadine and paracetamol	69 ± 6	68 ± 7	65 ± 8